Pure

University of Dundee

DOCTOR OF PHILOSOPHY

# HPV and p16 in head and neck cancer

Sailan, Ahmad Tarmidi

*Award date:*
2010

*Awarding institution:*
University of Dundee

Link to publication

**General rights**
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

**Take down policy**
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 17. Feb. 2017

University of Dundee

# HPV and p16 in head and neck cancer


**Ahmad Tarmidi Sailan**

BSc (Hons.)(Mal), SSn (UKMalaysia)


**A thesis submitted for the degree of Doctor in Philosophy**

**University of Dundee**

**October 2010**

**CONTENTS**


| | Page |
|---|---|
| **Table of contents** | i |
| **Dedication** | vii |
| **Acknowledgements** | viii |
| **Declaration** | ix |
| **Statement** | x |
| **Certificate** | xi |
| **List of Tables** | xii |
| **List of Figures** | xv |
| **List of Appendices** | xviii |
| **Abbreviations** | xx |
| **Summary** | xxiii |

**CHAPTER 1**

**INTRODUCTION**

| | |
|---|---|
| **1.1 ORAL CARCINOGENESIS** | 1 |
| **1.1.1 What is cancer?** | 1 |
| **1.1.2 Head and neck cancer** | 1 |
| **1.1.3 Oral cancer** | 3 |
| **1.1.4 Classification of oral cancer** | 3 |
| **1.1.5 Epidemiology of oral cancer** | 4 |
| **1.1.6 Aetiology of oral cancer** | 5 |
| **1.1.7 Risk factors for oral cancer** | 6 |
| 1.1.7.1 Tobacco smoking | 6 |
| 1.1.7.2 Smokeless tobacco | 7 |
| 1.1.7.3 Alcohol | 8 |
| 1.1.7.4 Synergy between smoking and alcohol | 12 |
| 1.1.7.5 Betel-quid or areca nuts (*Areca cathecu*) chewing | 12 |
| 1.1.7.6 Nutritional status and iron deficiency | 13 |
| 1.1.7.7 Bacteria and other microbial infections | 13 |
| 1.1.7.8 Viruses | 14 |
| **1.1.8 Clinical and Histopathological Aspects** | 15 |
| **1.1.9 Field Cancerisation versus Clonal Spread/Monoclonal Origin** | 18 |
| **1.1.10 Prognostic Factors** | 19 |
| 1.1.10.1 Tumour site | 21 |
| 1.1.10.2 Tumour thickness and invasion depth | 22 |
| 1.1.10.3 Tumour staging | 23 |
| 1.1.10.4 Extracapsular spread (ECS) | 23 |
| 1.1.10.5 Perineural invasion | 24 |
| 1.1.10.6 Pattern of infiltration | 24 |
| 1.1.10.7 Excision margins | 25 |
| **1.1.11 Oral precancer role** | 27 |
| 1.1.11.1 Dysplasia | 27 |
| **1.1.12 The clinical presentation of potentially malignant lesions** | 29 |
| **1.1.13 Potential malignant condition** | 29 |

**1.2 HUMAN PAPILLOMAVIRUS** ........ 31
**1.2.1 General information of HPV** ........ 31
**1.2.2 Types of HPV** ........ 31
**1.2.3 HPV oncoproteins** ........ 34
**1.2.4 Pathogenesis of HPV infection** ........ 35
**1.2.5 Detection and identification of HPVs** ........ 38
1.2.5.1 In cervical specimens ........ 39
1.2.5.2 In head and neck samples including oral sites ........ 39
**1.2.6 Meta-analysis of HPV involvement in specified cancer types** ........ 49
1.2.6.1 HPV in head and neck cancer ........ 49
**1.2.7 Reviews of head and neck cancer and the role of HPV in oral cancer** ........ 50
**1.2.8 Novel studies or reviews on HPVs involvement in various sites** ........ 51
1.2.8.1 HPVs in cutaneous squamous cell carcinomas ........ 51
1.2.8.2 HPVs in cervical squamous cell carcinomas ........ 52
1.2.8.3 HPV in oesophageal squamous cell carcinomas ........ 53
1.2.8.4 Cancer of the oropharynx ........ 53
1.2.8.5 Cancer of the larynx ........ 54
**1.3 p16$^{INK4a}$, A TUMOUR SUPPRESSOR GENE** ........ 55
**1.3.1 Genetic background** ........ 55
**1.3.2 p16 and oral cancer** ........ 57
**1.3.3 p16 promoter hypermethylation** ........ 58
**1.3.4 Was there a link between p16 expression and HPV infection?** ........ 62
**1.3.5 Is p16 as a surrogate marker for high risk- HPV in oral cancer?** ........ 64
**1.3.6 p16 immunohistochemistry and its semi-quantitative assessment** ........ 66
**1.4 HYPOTHESIS AND AIMS** ........ 69

**CHAPTER 2**

**MATERIALS AND METHODS**

**2.1 THE ROLE OF HPVs IN ORAL CANCER** ........ 71
**2.1.1 Introduction** ........ 71
**2.1.2 Aim** ........ 72
**2.1.3 General Equipment** ........ 73
**2.1.4 Specimens** ........ 75
2.1.4.1 Positive controls (*n*=2+3) ........ 75
2.1.4.2 Negative controls (*n*=2) ........ 75
2.1.4.3 Pilot Study ........ 76
2.1.4.4 The role of HPVs in oral disease progression (*n*=183) ........ 76
**2.1.5 Materials for MagneSil® Genomic, Fixed Tissue System, Cat. No. MD1490 (Promega Corporation, USA)** ........ 76
**2.1.6 Materials for QIAamp DNA FFPE Tissue Kit, Catalog no. 56404 (QIAGEN GmbH, Hilden, Germany)** ........ 77
**2.1.7 Reagents for Polymerase Chain Reaction (PCR)** ........ 78
**2.1.8 Materials for gel electrophoresis** ........ 79
**2.1.9 Materials for PCR Purification Kits** ........ 85
**2.1.10 Materials for automated PCR products sequencing** ........ 86
**2.1.11 Materials for SPF-DEIA** ........ 88
**2.1.12 Materials for $SPF_{10}$-$LiPA_{25}$ (version 1)** ........ 88
**2.1.13 Materials for Reverse Hybridisation Assay (RHA)** ........ 89

**2.1.14 $\alpha$ and $\beta$-HPV typing algorithm for pilot studies** ........ 90
**2.1.15 Tissue specimen preparation** ........ 92
**2.1.16 Isolating DNA from cells and tissues** ........ 92
**2.1.17 Human Genomic DNA Extraction from FFPET using a Promega kit** ........ 93
**2.1.18 Human Genomic DNA Extraction from FFPET using Qiagen kit** ........ 95
**2.1.19 Quantification and Qualification of Extracted DNA** ........ 97
**2.1.20 Agarose gel electrophoresis** ........ 98
2.1.20.1 Agarose gel preparation ........ 98
2.1.20.2 Gel Casting ........ 99
2.1.20.3 DNA sample loading ........ 99
2.1.20.4 Gel electrophoresis ........ 100
2.1.20.5 Gel staining using Fast Blast DNA Stain ........ 100
2.1.20.6 Gel image capturing ........ 101
**2.1.21 Optimisation of PCR** ........ 102
2.1.21.1 PCR for detection of human $\beta$-globin gene ........ 102
2.1.21.2 PCR for detection of protein L1 of mucosal HPV ........ 103
**2.1.22 PCR using extracted genomic DNA** ........ 104
2.1.22.1 The detection of $\beta$-globin gene (268 bp) ........ 104
2.1.22.2 Semi-nested PCR for detection of L1 gene of mucosal HPV ........ 105
2.1.22.3 Nested PCR for detection of L1 gene of general mucosal HPV ........ 106
2.1.22.4 Independent PCR for detection of L1 gene of mucosal HPV using two separate primer pairs ........ 106
**2.1.23 Purification of PCR products** ........ 107
2.1.23.1 QIAquick PCR Purification Kit ........ 107
2.1.23.2 QIAquick Gel Extraction Kit ........ 108
2.1.23.3 Quantification and qualification of the purified PCR products ........ 109
**2.1.24 Automated DNA Sequencing of the PCR products** ........ 110
2.1.24.1 Cycle Sequencing ........ 111
2.1.24.2 Purification of the extension products via ethanol/EDTA precipitation method ........ 112
2.1.24.3 Sample electrophoresis ........ 112
2.1.24.4 DNA sequencing outputs ........ 114
**2.1.25 SPF-DEIA and $SPF_{10}$-$LiPA_{25}$ (version 1) for alpha-HPV genotypes** ........ 115
2.1.25.1 SPF-DEIA ........ 115
2.1.25.2 $SPF_{10}$-$LiPA_{25}$ (version 1) ........ 116
**2.1.26 RHA for the Identification of beta HPV genotypes** ........ 118
2.1.26.1 PCR amplification ........ 118
2.1.26.2 HPV Genotyping ........ 119
**2.2 THE ROLE OF p16 IN ORAL DISEASE PROGRESSION** ........ 122
**2.2.1 Introduction** ........ 122
**2.2.2 Aim** ........ 123
**2.2.3 General Equipment** ........ 123
**2.2.4 Specimens for p16 immunohistochemistry staining** ........ 125
2.2.4.1 Optimisation of p16 immunohistochemistry ........ 125
2.2.4.2 Pilot study: Oral squamous cell carcinoma specimens for p16 IHC staining (n=60) ........ 125
2.2.4.3 The role of p16 in disease progression ........ 125
**2.2.5 Materials for immunohistochemistry staining** ........ 126
**2.2.6 The tissue specimens preparation** ........ 128
**2.2.7 p16 immunohistochemistry staining protocol** ........ 128
2.2.7.1 General protocol – Day 1 ........ 129
2.2.7.2 Pre-treatments – Day 1 ........ 131
2.2.7.3 General protocol ........ 133

**2.2.8 Optimisation of p16 expression** …… 135
2.2.8.1 Trial 1: Optimisation of p16 immunohistochemistry. …… 135
2.2.8.2 Trial 2: Comparing p16 primary antibody from two different suppliers…… 135
**2.2.9 Pilot study (n=60)** …… 136
**2.2.10 Oral disease progression study (n=183)** …… 136
**2.2.11 Semi-quantitative scoring for p16 immunohistochemistry staining** …… 136
**2.3 STATISTICAL ANALYSIS** …… 137

**CHAPTER 3**

**RESULT OF PILOT STUDIES**

**3.1 OVERVIEWS**…… 138
**3.2 OPTIMISATION OF DNA EXTRACTION**…… 139
**3.2.1 The quality and quantity of the extracted DNA of positive controls** …… 139
3.2.1.1 Repeated measurements within positive controls (HSGs) …… 139
3.2.1.2 Comparison between two positive controls (cells) …… 140
3.2.1.3 Preliminary conclusions …… 141
**3.2.2 The quality and quantity of the extracted DNA from FFPETs** …… 141
3.2.2.1 Repeated measurements within FFPETs …… 141
3.2.2.2 Comparison amongst FFPETs …… 142
3.2.2.3 Preliminary conclusions …… 143
**3.2.3 The effect of DNA volume to the DNA extraction** …… 144
3.2.3.1 DNA volume effect using Qiagen kit …… 144
3.2.3.2 DNA volume effect using Promega kit …… 144
3.2.3.3 Preliminary Conclusions …… 145
**3.2.4 DNA extraction methods comparison (Qiagen kit versus Promega kit)** …… 145
3.2.4.1 Comparison using extracted DNA from 3 coupes of FFPET …… 145
3.2.4.2 Comparison using extracted DNA from 5 coupes of FFPET …… 146
3.2.4.3 Comparison using extracted DNA from 10 coupes of FFPET …… 147
3.2.4.4 Preliminary conclusions …… 149
**3.3 OPTIMISATION OF POLYMERASE CHAIN REACTION**…… 150
**3.3.1 β-globin detection by PCR for DNA quality determination** …… 150
**3.3.2 PCR optimisation for L1 HPV detection** …… 151
3.3.2.1 First round Semi-nested/Nested PCR – MY11/MY09 primer pair (450 bp amplicons) …… 151
3.3.2.2 Second round Semi-nested PCR – MY11/GP6 primer pair (190 bp amplicons) …… 151
3.3.2.3 Second round Nested PCR – GP5+/GP6+ primer pair (140 bp amplicons) …… 154
3.3.2.4 Independent PCR reaction using GP5+/GP6+primer pair …… 155
**3.4 HPVs ROLE IN ORAL CANCER**…… 156
**3.4.1 Clinical Data**…… 156
**3.4.2 DNA extraction of 60 FFPETs** …… 159
**3.4.3 DNA quality determination by PCR for β-globin detection**…… 162
**3.4.4 Summary of the PCR amplification of L1 HPV gene detections**…… 162
**3.4.5 Purification of the PCR products** …… 163
**3.4.6 DNA sequencing of the PCR products**…… 166
**3.4.7 DNA sequences alignment** …… 168
**3.5 α-HPV GENOTYPING** …… 169
**3.6 β-HPV GENOTYPING** …… 172
**3.7 OVERALL HPV DETECTION** …… 173

**3.7.1 HPV prevalence and clinicopathological parameters** ........ 176

**3.7.2 Correlation among demographic and clinical parameters associated with HPVs** ........ 178

**3.7.3 Preliminary conclusions** ........ 183

**3.8 p16 ROLES IN ORAL CANCER** ........ 186

**3.8.1 Clinical data** ........ 186

**3.8.2 Semi-quantitative scoring for p16 immunohistochemistry** ........ 187

**3.8.3 Correlation among demographic and clinical parameters associated with p16** ........ 189

**3.9 CORRELATION BETWEEN HPVs AND p16 EXPRESSION** ........ 193

**3.10 SURVIVAL ANALYSIS** ........ 195

**3.10.1 Kaplan-Meier Survival Analysis** ........ 196

**3.10.2 Survival analysis according to the Cox Regression Model** ........ 200

**3.11 CONCLUSIONS** ........ 201


**CHAPTER 4**

**HPVs AND ROLE IN ORAL DISEASE PROGRESSION**


**4.1 OVERVIEWS** ........ 205

**4.2 CLINICAL DATA** ........ 205

**4.3 DNA EXTRACTION FROM ORAL DISEASE PROGRESSION STUDY** ........ 206

**4.4 β-GLOBIN DETECTION BY PCR FOR DNA QUALITY DETERMINATION** ........ 206

**4.5 SUMMARY OF THE PCR AMPLIFICATION OF L1 HPV GENE DETECTIONS** ........ 208

**4.6 MULTIPLE DNA SEQUENCES ALIGNMENT VIA BLASTn** ........ 210

**4.7 A COMPARISON OF HPV PREVALENCE IN BENIGN, DYSPLASIA AND CARCINOMA** ........ 210

**4.8 ODDS RATIO ANALYSIS AMONG DEMOGRAPHIC AND CLINICAL PARAMETERS BY HPV STATUS** ........ 222

**4.9 CORRELATION BETWEEN DEMOGRAPHIC AND CLINICAL PARAMETERS WITH HPV STATUS** ........ 226

**4.10 SURVIVAL ANALYSIS (N=80)** ........ 227

**4.10.1 Kaplan-Meier Survival Analysis** ........ 228

**4.10.2 Cox Regression Model** ........ 232

**4.11 CONCLUSIONS** ........ 232


**CHAPTER 5**

**p16 AND ORAL DISEASE PROGRESSION**


**5.1 OVERVIEW** ........ 235

**5.2 CLINICAL DATA** ........ 235

**5.3 RESULTS** ........ 236

**5.3.1 A comparison of p16 expression in benign, dysplasia and carcinoma** ........ 239

**5.3.2 A comparison of p16 positivity according to demographic and clinical parameters in each pathological group** ........ 243

**5.4 THE RISK FACTORS AMONG DEMOGRAPHIC AND CLINICAL PARAMETERS BY p16 STATUS** ........ 248

**5.5 CORRELATION BETWEEN DEMOGRAPHIC/CLINICOPATHOLOGICAL PARAMETERS AND p16 STATUS** 250

**5.6 SURVIVAL ANALYSIS ($N$ = 80)** ........ 251

**5.6.1 Kaplan-Meier Survival Analysis** ........ 251

**5.6.2 Cox Regression Model** ........ 252

**5.7 CONCLUSIONS** ........ 253

CHAPTER 6

CORRELATION BETWEEN HPVs AND p16 EXPRESSION IN HEAD AND NECK CANCER

6.1 OVERVIEW ………… 255
6.2 CLINICAL DATA ………… 255
6.3 HPVs EXPRESSION IN ORAL MUCOSA ………… 257
6.4 p16 EXPRESSION IN ORAL MUCOSA ………… 263
6.5 CORRELATION ANALYSES ………… 266
6.6 CONCLUSIONS ………… 274

CHAPTER 7

THE PROGNOSTIC IMPLICATION OF HPV AND p16

7.1 OVERVIEW ………… 277
7.2 SURVIVAL ANALYSIS AND COX PROPORTIONAL HAZARD MODELS ………… 277
7.3 CONCLUSIONS ………… 296

CHAPTER 8

DISCUSSION

8.1 INTRODUCTION ………… 300
8.2 HPV PREVALENCE IN THE HEAD AND NECK CANCER ………… 303
8.3 p16 EXPRESSION IN THE HEAD AND NECK CANCER ………… 309
8.4 CORRELATION AMONG DEMOGRAPHIC AND CLINICOPATHOLOGICAL PARAMETERS, HPV AND p16 EXPRESSION ………… 312
8.5 RISK ASSESSMENT WITH DEMOGRAPHIC AND CLINICOPATHOLOGICAL PARAMETERS ASSOCIATED WITH HPV AND p16 STATUS ………… 315
8.6 SURVIVAL ANALYSIS AND PROGNOSTIC IMPLICATIONS OF HPV AND p16 ………… 317

CHAPTER 9

CONCLUSIONS ………… 319

REFERENCES ………… 322
APPENDICES ………… 369
PUBLICATIONS ………… 444

## DEDICATION

*To*

*My wife, Zulaikha for her persistent prayers and support*
*My beloved parents who have still believe in me*
*My deepest and sweet memories to my late father and sisters,*
*Haji Sailan, Pauziah and Hamizah*
*…..and to the rest of my family members for both sides*

**ACKNOWLEDGEMENTS**

Dr. Michaelina Macluskey, for the last three years I've been under her supervision but I've still unable to pick up appropriate words which describe her best.
I've gained from her not only an invaluable knowledge about research but also how to maintain higher professionalism during prolonged endurance throughout my PhD course especially related to communications. Thus far, I've learned on how to have a good rapport among colleagues. My future prospect students would be benefited of the positive attitudes that I had learned.

I would like to thank Dr. John Gibson, Professor Peter Mossey, Dr. Karin Purdie, Mr. Maurits de Koning, Dr. Sharon White, Dr. Grant McIntyre, Mr. Phillip McLoughlin, Dr. Sarah Jones and Dr. Ian Ellis for their advice.

My appreciation also goes to staff of Unit of Oral Surgery and Medicine, Mrs Eileen Black. My thanks also to Mrs Jacqueline Cox, Mrs Valerie Wilson, Ms Margaret Florence, Mr Paul Hughes and Mr Simon Scott

Last but not least deepest gratitude to the government of Malaysia for giving me an opportunity to pursue my study especially at University of Dundee

## DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ………………………………………………….. Candidate

Date ……………………….

**STATEMENT 1**

This thesis is being submitted in fulfilment for the requirements for the degree of Doctor of Philosophy.

Signed ………………………………………….. Candidate

Date …………………………….

**STATEMENT 2**

This thesis is the result of my own independent work and investigation except where otherwise stated. Other sources are acknowledged by footnotes giving references. A bibliography is appended.

Signed ………………………………………….. Candidate

Date …………………………….

**STATEMENT 3**

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisation.

Signed ………………………………………….. Candidate

Date …………………………….

# CERTIFICATE

We hereby certify that Ahmad Tarmidi Sailan has fulfilled the conditions of Ordinance 39 of the University of Dundee and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Signature ……………………………… Date ………………………….

Dr. Michaelina Macluskey
Unit of Oral Surgery and Medicine
Dundee Dental Hospital and School
College of Medicine, Dentistry and Nursing
University of Dundee

Signature ……………………………… Date ………………………….

Dr. John Gibson
Unit of Oral Surgery and Oral Medicine
Dundee Dental Hospital and School
College of Medicine, Dentistry and Nursing
University of Dundee

Signature ……………………………… Date ………………………….

Professor Peter A. Mossey
Department of Orthodontics
Dundee Dental Hospital and School
College of Medicine, Dentistry and Nursing
University of Dundee

## LIST OF TABLES

**CHAPTER 1** Page

Table 1.1: Selected findings on alcohol with other risk factors in oral cancer ……. 10
Table 1.2: Reported evidence on alcohol with others as the risk factors in oral cancer ……. 11
Table 1.3: TNM stage grouping for oral cancer …… 17
Table 1.4: Polyclonal origin versus Monoclonal origin …… 18
Table 1.5: The main role of HPV proteins …… 32
Table 1.6: Human HPV supergroups and selected candidates …… 33
Table 1.7: Epidemiologic classification of HPV types in alpha HPV supergroup …… 34
Table 1.8: HPV prevalence among studies (2000-2010) in normal individuals …… 40
Table 1.9: HPV prevalence among studies (2000-2010) in individuals with oral benign …… 41
Table 1.10: HPV prevalence among studies (2000-2010) in individuals with oral dysplastic …… 41
Table 1.11: HPV prevalence among studies (2000-2010) in individuals with carcinoma …… 41
Table 1.12: Comparison among various studies for HPV detection in cervical specimens …… 42
Table 1.13: Comparison among various methods for HPV detection and genotyping in the head and neck …… 47
Table 1.14: The percentage of p16 expression among studies …… 58
Table 1.15: The prevalence of p16 promoter hypermethylation among studies …… 61
Table 1.16: An association observed between p16 (IHC) and HPV (HR) expression among studies …… 65
Table 1.17: No association observed between p16 (IHC) and HPV (HR) expression among studies …… 65
Table 1.18: Comparison of p16 IHC semi-quantitative scoring amongst studies …… 67

**CHAPTER 2**

Table 2.1 Oligonucleotide primers used for HPV detection …… 81
Table 2.2 Reagents and their composition for each PCR amplification …… 82
Table 2.3 PCR condition for each amplification method …… 83
Table 2.4 Reagents for cycle sequencing of PCR products …… 111
Table 2.5 Cycle sequencing condition of each sample …… 112
Table 2.6 Resuspension volumes for reaction plates …… 113

**CHAPTER 3**

Table 3.1a: The effect of duplication …… 139
Table 3.1b: The effect of replication …… 140
Table 3.2a: Comparison between two positive controls …… 140
Table 3.2b: The effect of replication …… 141
Table 3.3a: The effect of duplication …… 142
Table 3.3b: The effect of replication …… 142
Table 3.4a: Comparison amongst randomly selected six FFPETs …… 143
Table 3.4b: The effect of replication …… 143
Table 3.5: Mean comparison between two kits (3 coupes) …… 146
Table 3.6: Mean comparison between two kits (5 coupes) …… 147
Table 3.7: Mean comparison between two kits (10 coupes) …… 148
Table 3.8: Summary of the comparison between extraction kits …… 149
Table 3.9: Clinical details of oral cancer patients …… 157
Table 3.10: Clinicopathologic parameters of oral cancer patients …… 158

Table 3.11: The purity and yield of the extracted DNA based on parameters ………. 160

Table 3.12: *t*-Test and ANOVA for means comparison ………. 161

Table 3.13: Mann-Whitney and Kruskal Wallis for means comparison ………. 161

Table 3.14: PCR positivity for L1 HPV gene detection by PCR types ………. 162

Table 3.15: Means comparison of the purified amplicons and kit used ………. 163

Table 3.16: *t*-Test and ANOVA ………. 166

Table 3.17: Mann-Whitney and Kruskal-Wallis Test ………. 166

Table 3.18: The summary of alpha HPV types detected via PCR and sequencing ………. 168

Table 3.19: The summary of alpha HPV types detected by via $SPF_{10}$-$LiPA_{25}$ (version 1) ………. 170

Table 3.20: Alpha HPV types detected via the combination results of two methods ………. 171

Table 3.21: Beta HPV types detected via PM-PCR and RHA methods ………. 172

Table 3.22: Overall HPV types detected by using the results of all methods ………. 175

Table 3.23: Normally distributed data assumption among parameters ………. 177

Table 3.24: Demographic and risk factor of oral cancer by HPV status ………. 178

Table 3.25: Clinicopathologic characteristics of oral cancer by HPV status ………. 179

Table 3.26: The distribution of HPV types within demographic characteristics ………. 180

Table 3.27: The distribution of HPV types within clinicopathological parameters ………. 181

Table 3.28: The correlations between demographic/clinical parameters and HPV status ………. 182

Table 3.29: Demographic and risk factor characteristics of oral caner by p16 status ………. 190

Table 3.30: Clinicopathologic characteristics of oral cancer by p16 status ………. 191

Table 3.31: The correlations between demographic/clinical parameters and p16 status ………. 192

Table 3.32: Cross-tabulation of HPV versus p16 status ………. 193

Table 3.33: Chi-square analysis of prevalence of p16 positivity with HPV status ………. 194

Table 3.34: Chi-square analysis of prevalence of p16 staining intensity with HPV status ………. 194

Table 3.35: The prognostic significance from overall survival analysis ………. 197

Table 3.36: Patient survival analysis by Cox regression model ………. 201


**CHAPTER 4**


Table 4.1: Clinical details of patients in each pathological group ………. 207

Table 4.2: Histopathological details of patients with carcinoma ………. 208

Table 4.3: Overall PCR results for L1 HPV gene detection ………. 209

Table 4.4: Summary of PCR results of L1 HPV gene detection (pathological groups) ………. 209

Table 4.5: Summary of HPV types detected in each pathological group ………. 212

Table 4.6: The distribution of HPV types within clinical groups in benign (N=43) ………. 216

Table 4.7: The distribution of HPV types within clinical groups in dysplasia (N=5) ………. 217

Table 4.8: The distribution of HPV types within clinical groups in carcinoma (N=42) ………. 218

Table 4.9: The distribution of HPV types within histopathological parameters in carcinoma group (N=42) ………. 219

Table 4.10: Demographic and risk factor characteristics of oral cancer by HPV status in each pathological group ………. 223

Table 4.11: Demographic and risk factor characteristics of oral cancer by high risk-HPV status in each pathological group ... 224

Table 4.12: Histopathological risk factor properties by HPV status in carcinoma group ………. 225

Table 4.13: Histopathological risk factor properties by high risk-HPV status in carcinoma group ………. 225

Table 4.14: The prognostic significance from overall survival analysis in carcinoma patients ………. 228

Table 4.15: The summary of the survival analysis done by Cox regression ………. 232

**CHAPTER 5**


Table 5.1: Clinical details of oral cancer patients …….. 236
Table 5.2: Kruskal-Wallis and Mann-Whitney tests on p16 positivity …….. 240
Table 5.3: Kruskal-Wallis and Mann-Whitney tests on p16 staining intensity ……. 241
Table 5.4: A comparison of p16 positivity in each pathological group ……. 245
Table 5.5: A comparison of p16 staining intensity in each pathological group …… 246
Table 5.6: A comparison of p16 positivity in carcinoma group only …… 247
Table 5.7: A comparison of p16 staining intensity in carcinoma group only …… 247
Table 5.8: Demographic and risk factor characteristics by p16 status in each pathological group ……. 249
Table 5.9: Histopathological risk factor properties by p16 status in carcinoma group ……. 250
Table 5.10: The prognostic significance from overall survival analysis in carcinoma patients …….. 252
Table 5.11: The summary of the survival analysis done by Cox regression …… 252


**CHAPTER 6**


Table 6.1: Clinical details of oral cancer patients …….. 256
Table 6.2: Overall HPV types detected in oral carcinoma (N=140) ……. 257
Table 6.3: The distribution of HR-HPV and LR-HPV types within demographic characteristics …… 261
Table 6.4: Significant correlations within demographic and clinical parameters, HPVs and p16 status …….. 267
Table 6.5: Significant correlations between demographic and clinical parameters; HPVs and p16 status ……... 270
Table 6.6: Correlation analyses between HPVs and p16 status ……. 271
Table 6.7: Correlation analyses between p16 positivity and HPV status ……... 272
Table 6.8: Cross-tabulation of HPV versus p16 expression …….. 273


**CHAPTER 7**


Table 7.1: The prognostic significance risk factors on OS from Kaplan-Meier test …….. 279
Table 7.2: The prognostic significance risk factors on RFS from Kaplan-Meier test …… 280
Table 7.3: Univariate and multivariate of OS in total patients (N=140) …… 282
Table 7.4: Univariate and multivariate of RFS in total patients (N=140) ……. 283
Table 7.5: Demographic and risk factor characteristics of head and neck cancer by p16 status …….. 284
Table 7.6: Prognostic significance of risk factors of HPVs by p16 status ……. 285
Table 7.7: Adjusted hazard ratios[a] for p16/HPV models of survival and recurrence …… 286

## LIST OF FIGURES


**CHAPTER 1** Page

Figure 1.1: Gene alteration results of tobacco smoking ………. 7
Figure 1.2: Prognostic factors interaction leading to appropriate prognosis ………. 20
Figure 1.3: Genome map of HPV16 ………. 32
Figure 1.4: p16 roles in cell cycle regulation ………. 56
Figure 1.5: The postulated mechanism on upregulation of p16 in HPV infection ………. 62
Figure 1.6: The overall strategies of the study ………. 70

**CHAPTER 2**

Figure 2.1: General instruments ………. 74
Figure 2.2: Instruments for PCR and RHA ………. 80
Figure 2.3: Instruments for gel electrophoresis ………. 84
Figure 2.4: Instruments for automated DNA sequencing ………. 87
Figure 2.5: α-HPV and β-HPV detection and genotyping algorithm ………. 91
Figure 2.6: GH20 and PC04 primer annealing sites in human beta globin gene ………. 102
Figure 2.7: Primer annealing sites for universal primer sets and type specific SPF primer sets and probes in HPV 6 L1 protein ………. 104
Figure 2.8: The target region in E1 protein gene for beta HPV genotyping ………. 118
Figure 2.9: ABC immunochemical staining procedure ………. 122
Figure 2.10: Motic BA400 Biological Microscope ………. 124
Figure 2.11: Key optimisation steps for p16 expression ………. 130

**CHAPTER 3**

Figure 3.1: β-globin detection in 2% agarose gel ………. 150
Figure 3.2: The effect of DNA template concentration in 2% agarose gel ………. 151
Figure 3.3: $MgCl_2$ effect is visualised in 2% agarose gel ………. 152
Figure 3.4: The effect of DNA template concentration in 2% agarose gel ………. 153
Figure 3.5: The effect of undiluted 450 bp amplicons (as the template for second round of semi-nested PCR) in 2% agarose gel ………. 153
Figure 3.6: Effect of diluted 450 bp amplicons (as the template for second round of semi-nested PCR) in 2% agarose gel 154
Figure 3.7: 2% agarose gel electrophoresis of diluted 450 bp amplicons as the template for the second round of nested PCR ………. 154
Figure 3.8: PCR reamplification effects of 140 bp amplicons without removing samples from thermocycler ………. 155
Figure 3.9: The effect of DNA template concentration in 2% agarose gel ………. 156
Figure 3.10: DNA purity (A) and DNA yield (B) comparisons among PCR products ………. 164
Figure 3.11: DNA purity (A) and DNA yield (B) comparisons between purification kits ………. 165
Figure 3.12: Selected automated DNA sequencing results ………. 167
Figure 3.13: Schematic representation of DNA contig formation ………. 167
Figure 3.14: LR α-HPV and HR α-HPV detected via PCR and DNA sequencing ………. 169
Figure 3.15: LR α-HPV and HR α-HPV detected via $SPF_{10}$-$LiPA_{25}$ (version 1) ………. 170
Figure 3.16: LR α-HPV and HR α-HPV detected via both methods ………. 171
Figure 3.17: The status of β-HPV types detected ………. 173

Figure 3.18: The total of HPV positively detected in this pilot study ……………………………………………….....…… 174
Figure 3.19: The distribution of HPV types detected …………………………………………………………………….. 174
Figure 3.20: The postulated mode of HPV infection ……………………………………………………………………... 175
Figure 3.21: The distribution of HPV detected …………………………………………………………………………… 176
Figure 3.22: Schematic representation of correlation plot amongst parameters ………………………………………….. 185
Figure 3.23: Photomicrograph of p16 immunohistochemical staining …………………………………………………… 188
Figure 3.24: Photomicrograph of p16 immunohistochemical staining intensity ……………………………………………. 188
Figure 3.25: The total score of p16 IHC in this pilot study ……………………………………………………………… 189
Figure 3.26: An association amongst anatomic sites and overall survival as generated by Kaplan-Meier test ……………. 197
Figure 3.27: An association amongst tumour sizes and overall survival as generated by Kaplan-Meier test ………………. 198
Figure 3.28: An association amongst tumour stage and overall survival as generated by Kaplan-Meier test ……………… 198
Figure 3.29: An association between patients with alcohol exposure and overall survival as generated by Kaplan-Meier test …………………………………………………………………………………………………… 199
Figure 3.30: An association amongst smoking-alcohol exposure and overall survival as generated by Kaplan-Meier test ... 199
Figure 3.31: An association between patients with positive β-HPV types and overall survival as generated by Kaplan-Meier test ……............................................................................................................................................... 200

**CHAPTER 4**

Figure 4.1: Overall HPV types positively detected in each pathological group …..…………………………………….. 213
Figure 4.2: HPV types distributions in each pathological group ……………………..…………………………………….. 213
Figure 4.3: The breakdown of specific HPV types (%) identified ………………………………………………………. 214
Figure 4.4: An association between gender and overall survival as generated by Kaplan-Meier test ……………………... 229
Figure 4.5: An association between age group and overall survival as generated by Kaplan-Meier test ………………….. 229
Figure 4.6: An association among anatomic sites and overall survival as generated by Kaplan-Meier test ……………… 230
Figure 4.7: An association among tumour sizes and overall survival as generated by Kaplan-Meier test ……….………... 230
Figure 4.8: An association among regional lymph nodes and overall survival as generated by Kaplan-Meier test ……..... 231
Figure 4.9: An association among histological grade and overall survival as generated by Kaplan-Meier test …………... 231

**CHAPTER 5**

Figure 5.1: Photomicrograph of p16 staining across pathological groups …………………………………………………. 237
Figure 5.2: Overall p16 status in each pathological group ……………………………………………………………… 238
Figure 5.3: The frequency of p16 staining intensity in each pathological group ……………………………………….. 238
Figure 5.4: Photomicrograph of p16 staining across histological grade …………………………………………………… 242
Figure 5.5 An association between p16 positivity and overall survival as generated by Kaplan-Meier test ……………... 251

**CHAPTER 6**

Figure 6.1: The total of HPV positively detected in the pooled data of oral carcinomas ………………………………….. 258
Figure 6.2: The distribution of HPV types detected. ………………………………………………………………….. 258
Figure 6.3: The distribution of HPV types detected …...........…………………………………………………………….. 259
Figure 6.4: Overall p16 positivity in this cohort ………………………………………………………………………… 265
Figure 6.5 Overall p16 staining intensity in this cohort ………………………………………………………………… 265
Figure 6.6 Schematic representation of correlations within demographic and clinicopathological parameters. …………. 268
Figure 6.7 Schematic representation of correlations within HPV status ………………………………………………... 268
Figure 6.8 Schematic representation of correlations within demographic and clinicopathological parameters. …………. 269

Figure 6.9: Schematic representation of correlations between demographic/clinicopathological parameters and p16 status. …………………………………………………………………………………………………………… 271

**CHAPTER 7**

Figure 7.1 Total patients, significantly worse OS noted in patients with higher intensity of p16 staining (2+/3+) compared with those with p16 negative/weak staining (1+) …………………………………………………... 281
Figure 7.2A OS by HPV/p16 status as generated by Kaplan-Meier test………………………………………………….. 292
Figure 7.2B RFS by HPV/p16 status as generated by Kaplan-Meier test. ………………………………………………….. 292
Figure 7.3A OS by HPV16/p16 status as generated by Kaplan-Meier test. ………………………………………………… 293
Figure 7.3B RFS by HPV16/p16 status as generated by Kaplan-Meier test. ……………………………………………….. 293
Figure 7.4A OS by HPV/p16 staining intensity as generated by Kaplan-Meier test. ……………………………………….. 294
Figure 7.4B RFS by HPV/p16 staining intensity as generated by Kaplan-Meier test. ……………………………………… 294
Figure 7.5A OS by HPV16/p16 staining intensity as generated by Kaplan-Meier test. …………………………………….. 295
Figure 7.5B RFS by HPV16/p16 staining intensity as generated by Kaplan-Meier test. …………………………………… 295

**LIST OF APPENDICES**


| | | Page |
|---|---|---|
| Appendix 1 | PCR reagents preparation ………………………………………………………………………….. | 369 |
| Appendix 2 | Positive Control DNAs ……………………………………………………………………………… | 370 |
| Appendix 3 | Reagents preparation for Fast Blast DNA stain ………………………………………………………… | 371 |
| Appendix 4 | Human β-globin DNA sequence ………………………………………………………………….. | 372 |
| Appendix 5 | Base-nucleic acid codes ……………………………………………………………………………… | 373 |
| Appendix 6 | Table A. Quality and quantity of extracted DNA within positive controls (HSGs) …………………… | 374 |
| | Table B. Comparison between positive controls (HSGs and 3T3) …………………………………….. | 374 |
| Appendix 7 | Table A. Quality and quantity of extracted DNA within randomly selected FFPETs …………………. | 375 |
| | Table B. Comparison between randomly selected 6 FFPETs ………………………………………….. | 375 |
| Appendix 8 | DNA quantification of selected five FFPETs using QIAGEN kit (QIAamp DNA FFPE) ……………. | 376 |
| Appendix 9 | One-way ANOVA on extracted DNA using Qiagen kit ………………………………………………. | 377 |
| | Post Hoc Test: Multiple Comparisons ……………………………………………………………….. | 378 |
| Appendix 10 | DNA quantification of selected five FFPETs using PROMEGA kit (Magnesil Genomic) …………… | 379 |
| Appendix 11 | One-way ANOVA on extracted DNA using Promega kit …………………………………………….. | 380 |
| Appendix 12 | DNA qualification and quantification comparison between QIAGEN kit (QIAamp DNA FFPE) and PROMEGA kit (Magnesil Genomic) ………………………………..……………………………….. | 381 |
| Appendix 13 | Quantification of 60 extracted DNA (QIAamp FFPE DNA kit) …….………………………………… | 382 |
| Appendix 14A | Positive PCR amplification results for β-globin and L1 of alpha HPV ………………….…………… | 383 |
| Appendix 14B | PCR amplification results for β-globin (positive) and L1 of α-HPV (negative) ……………………….. | 384 |
| Appendix 15 | DNA quantification of the PCR products prior automated DNA sequencing ………………………….. | 385 |
| Appendix 16 | Automated DNA sequencing results (actual size obtained from contig) ……………………………….. | 386 |
| Appendix 17 | CLUSTAL W (1.83) multiple sequences alignment of L1 HPVs ……………………………………… | 387 |
| Appendix 18 | Results of the DNA sequencing alignment of L1 α-HPV ……………………………………………… | 388 |
| Appendix 19 | α-HPV genotyping: Raw data of SPF-DEIA analysis (microtiter plate layout) ……………………….. | 389 |
| Appendix 20 | α-HPV genotyping: Raw data of $SPF_{10}$-$LiPA_{25}$ (version 1) …………………………………….…… | 390 |
| Appendix 21 | Interpretation sheet for α-HPV genotyping: $SPF_{10}$-LiP $A_{25}$ (version 1) ……………………………….. | 392 |
| Appendix 22 | Alpha HPV genotyping by using $SPF_{10}$-$LiPA_{25}$ (version 1) …………………………………………… | 393 |
| Appendix 23 | Overall alpha HPV types detected by several methods in pilot study samples ………………………… | 394 |
| Appendix 24 | Raw data - Beta HPV genotyping ………………………………………………………………………. | 395 |
| Appendix 25 | DIASSAY Skin (beta) HPV genotyping strip ……………………………………………………….... | 398 |
| Appendix 26 | Beta HPV types detected by PM-PCR and RHA methods in pilot study samples ……………………... | 399 |
| Appendix 27 | Overall α- and β-HPV types detected by several methods in pilot study samples ……………………... | 400 |
| Appendix 28 | The association of tobacco smoking and alcohol consumption with HPV status ………………………. | 401 |
| Appendix 29 | Semi-quantitative p16 immunohistochemistry staining ………………………………………………… | 402 |
| Appendix 30 | (A) Clinical and histopathology parameters of 176 DNA samples for oral disease progression study .... | 403 |
| | (B) Clinical and histopathology parameters of 7 DNA samples (normal tissues) ................…………... | 406 |
| | (C) DNA purity and DNA yield of 7 extracted DNA samples (normal tissues) ..................…………... | 406 |
| | (D) PCR results for β-globin and L1 α–HPV (normal tissues) ..........………........................................ | 406 |
| | (E) DNA sequencing and multiple sequences alignment results (normal tissues) ...............…………... | 406 |
| Appendix 31 | DNA purity and DNA yield of 84 extracted DNA samples (Benign) ……………………………..…… | 407 |
| Appendix 32 | DNA purity and DNA yield of 12 extracted DNA samples (Dysplasia) ……………………………..…… | 409 |
| Appendix 33 | DNA purity and DNA yield of 80 extracted DNA samples (Carcinoma) …………………………...…. | 410 |
| Appendix 34 | PCR results for β-globin and L1 of alpha HPV (Benign) ………………………………………………. | 412 |
| Appendix 35 | PCR amplification results for β-globin and L1 of alpha HPV (Dysplasia) …………………………….. | 414 |
| Appendix 36 | PCR results for β-globin and L1 of alpha HPV (Carcinoma) …………………………………………... | 415 |

| | | |
|---|---|---|
| Appendix 37 | DNA sequencing and multiple sequences alignment results (Benign) …………………………………. | 417 |
| Appendix 38 | DNA sequencing and multiple sequences alignment results (Dysplasia) ………………………………. | 418 |
| Appendix 39 | DNA sequencing and multiple sequences alignment results (Carcinoma) …………………………... | 419 |
| Appendix 40 | Table A: ANOVA and Independent-samples *t*-Test for means comparison ………………………..... | 420 |
| | Table B: Kruskal-Wallis and Mann-Whitney Tests …………………………………………………….. | 420 |
| Appendix 41 | Table A: Means comparison of the purified amplicons and kit used in benign samples ……………….. | 421 |
| | Table B: Independent-samples *t*-Test for means comparison (Benign) ……………………………... | 421 |
| | Table C: Mann-Whitney Test for means comparison (Benign) ……………………………………… | 421 |
| Appendix 42 | Table A: Means comparison of the purified amplicons and kit used in dysplasia samples …………….. | 422 |
| | Table B: ANOVA and Independent-samples *t*-Test for means comparison (Dysplasia) ………………. | 422 |
| | Table C: Kruskal-Wallis and Mann-Whitney Tests (Dysplasia) …………………………………….. | 422 |
| Appendix 43 | Table A: Means comparison of the purified amplicons and kit used in carcinoma samples …………… | 423 |
| | Table B: ANOVA and Independent-samples *t*-Test for means comparison (Carcinoma) ……………... | 423 |
| | Table C: Kruskal-Wallis and Mann-Whitney Tests (Carcinoma) …………………………………… | 423 |
| Appendix 44 | Table A: Crosstabs with Chi-square and Phi analyses of HPV positivity among clinicopathological parameters ……………............................................................................................................ | 424 |
| | Table B: Crosstabs with Chi-square and Cramer'V analyses of HPV positivity among clinicopathological parameters ……………...................................................................................... | 425 |
| Appendix 45 | Table A: Crosstabs with Chi-square and Phi analyses of alpha HPV positivity among clinicopathological parameters ………………................................................................................. | 426 |
| | Table B: Crosstabs with Chi-square and Cramer'V analyses of alpha HPV positivity among clinicopathological parameters ……………...................................................................................... | 427 |
| Appendix 46 | Table A: Crosstabs with Chi-square and Phi analyses of Low risk α-HPV positivity among clinicopathological parameters ………….......................................................................................... | 428 |
| | Table B: Crosstabs with Chi-square and Cramer'V analyses of Low risk α-HPV positivity among clinicopathological parameters ……………...................................................................................... | 429 |
| Appendix 47 | Table A: Crosstabs with Chi-square and Phi analyses of High risk α-HPV positivity among clinicopathological parameters ………….......................................................................................... | 430 |
| | Table B: Crosstabs with Chi-square and Cramer'V analyses of High risk α-HPV positivity among clinicopathological parameters ……………...................................................................................... | 431 |
| Appendix 48 | Table A: Pearson's correlation between clinical parameters and HPV in benign ……………………… | 432 |
| | Table B: Spearman's correlation between clinical parameters and HPV in benign ……………………. | 432 |
| Appendix 49 | Table A: Pearson's correlation between clinical parameters and HPV in dysplasia …………………… | 433 |
| | Table B: Spearman's correlation between clinical parameters and HPV in dysplasia …………………. | 433 |
| Appendix 50 | Table A: Pearson's correlation between clinical parameters and HPV in carcinoma ………………….. | 434 |
| | Table B: Spearman's correlation between clinical parameters and HPV in carcinoma ………………... | 434 |
| Appendix 51 | Table A: Pearson's correlation between clinical parameters and p16 status ……………….................. | 435 |
| | Table B: Spearman's correlation between clinical parameters and p16 status ……………….................. | 435 |
| Appendix 52 | Table A: Pearson's correlation between clinical parameters and p16 staining status ………………….. | 436 |
| | Table B: Spearman's correlation between clinical parameters and p16 staining status ………………... | 436 |
| Appendix 53 | Table A: Crosstabs with Chi-square test of HPV status among clinicopathological parameters ………. | 437 |
| | Table B: Crosstabs with Chi-square test and Cramer's V of p16 status among clinicopathological parameters ………………………………………………………………………………………… | 439 |
| | Table C: Crosstabs with Cramer's V test of HPV status among clinicopathological parameters ……… | 440 |
| | Table D: Crosstabs with Cramer's V test of p16 status among clinicopathological parameters ………. | 442 |
| Appendix 54 | The overall of DNA purity and DNA yield of extracted DNA ………………………………………… | 443 |

# Abbreviations

| | |
|---|---|
| ABP | 4-4-aminobiphenyl |
| ADH | Alcohol dehydrogenase |
| AhR | Aryl hydrocarbon receptor |
| ALDH | Aldehyde dehydrogenases |
| AJCC | American Joint Committee on Cancer |
| ANOVA | Analysis of Variance |
| ARE | Antioxidant response element |
| AKR | Aldo-keto reductase |
| ARK | Alveolar ridge keratoses |
| ASCUS | Atypical squamous cells of undetermined significance |
| BLAST | Basic Local Alignment Systematic Tools |
| BPDE | Benzo[a]pyrene diolepoxide |
| BS-HPV | Broad spectrum-HPV |
| BS-RLB | Broad spectrum-Reverse Line Blot |
| cDNA | Complementary DNA |
| CI | Confidence interval |
| CIN | Cervical intraepithelial neoplasia |
| Cis | Carcinoma in situ |
| CDKN2A | Cyclin dependent kinase inhibitor |
| CM-LA | Cytyc media-based linear array HPV test |
| CSA-ISH | In situ hybridisation with signal amplification |
| CTAB | Hexadecyltrimethyl ammonium bromide |
| CYP2E1 | P450-related enzyme |
| DG | Digene HPV genotyping |
| DM-LA | Digene media-based linear array HPV test |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphates |
| $dH_2O$ | Sterile distilled water |
| ddNTP | dideoxyribonucleotide triphosphates |
| DFS | Disease-free survival |
| DRZ | Diagonal radioactive zone |
| DSS | Disease-specific survival |
| EBV | Epstein-Barr virus |
| ECA | Endocervical adenocarcinomas |
| ECS | Extracapsular spread |
| EDTA | Ethylenediaminetetraacetic acid |
| EMA | Endometrial adenocarcinomas |

| | |
|---|---|
| E2F | Elongation factor |
| E2F-DB | Elongation factor-DB |
| EV | Epidermodysplasia verruciformis |
| FISH | Fluorescence In Situ Hybridisation |
| FFPETs | Formalin-fixed paraffin-embedded tissues |
| GP-RLB | GP5+ or GP6+ -based Reverse Line Blot |
| HC | Hybrid captured |
| H & E | Hematoxylin and Eosin |
| HGS | Human genetic signatures |
| HHV8 | Human herpesvirus 8 |
| HNSCC | Head and neck squamous cell carcinoma |
| HPV | Human papillomavirus |
| HRs | Hazard ratios |
| HR-HPV | High risk-Human papillomavirus |
| HRP | Horseradish peroxidise |
| HSD | Honestly significant differences |
| HSG | Human genetic signatures |
| HSPGs | Heparan sulphate proteoglycans |
| H-SIL | High grade squamous intraepithelial lesion |
| ICD | International Classification of Diseases |
| IHC | Immunohistochemistry |
| IG E CE | INNO-LiPA HPV Genotyping Extra CE assay |
| INK | Inhibitor of cyclin – dependent kinase |
| ISH | In situ hybridisation |
| ITF | Invasive tumour front |
| LBC | Liquid-based cytology |
| LCR | Long control region |
| LiPA | Line Probe Assay |
| L-SIL | Low grade squamous intraepithelial lesion |
| MA | Marked cytological atypia |
| MEC | Mucoepidermoid carcinoma |
| MP | Maras powder |
| MPG | Multiplexed HPV genotyping |
| MS-PCR | Methylation specific-PCR |
| Nrf2 | Nuclear respiratory factor2 |
| NSND | Non-smoking and non-drinking |
| OL | Oral lesion |
| OLP | Oral lichen planus |
| OPSCC | Oropharyngeal squamous cell carcinoma |
| OR | Odds ratio |

| | |
|---|---|
| ORF | Open Reading Frame |
| OS | Overall Survival |
| OSCC | Oral squamous cell carcinoma |
| OSF | Oral submucous fibrosis |
| PAHs | Polynuclear aromatic hydrocarbons |
| PCR | Polymerase Chain Reaction |
| PDZ | Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) |
| PH | Promoter hypermethylation |
| PIN | Penile intraepithelial neoplasia |
| PM-PCR RHA | Broad spectrum PCR and reverse hybridisation assay |
| Rb | Retinoblastoma |
| RE | Restriction enzyme |
| RFS | Reccurrence-Free Survival |
| RFLP | Random fragment length polymorphism |
| RFMA | Restriction fragment methylation analysis |
| RHA | Reverse Hybridisation Assay |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcriptase-PCR |
| SDS | Sodium dodecyl sulphate |
| $SPF_{10}$-LiPA | Short PCR Fragment-Line Probe Assay |
| SPSS | Statistical Package for Social Sciences |
| SSCP | Single strand conformation polymorphism |
| STK | Smokeless Tobacco Keratosis |
| STM | Specimen transport medium |
| STWS | Scott's Tap Water Solution |
| TAE | Tris-Acetate-EDTA |
| TBE | Tris-Borate-EDTA |
| TGFb | Tumour growth factor |
| TILs | Tumour-infiltrating lymphocytes |
| TNM | T, tumour size; N, node involvement; M, distant metastasis |
| TSGs | Tumour suppressor genes |
| UICC | Internationale Contre le Cancer |
| URR | Upstream regulatory region |
| UV | Ultra violet |
| VAIN | Vaginal intraepithelial neoplasia |
| VC | Verracous carcinoma |
| VIN | Vulval intraepithelial neoplasia |
| WHO | World Health Organisation |
| XRE | Xenobiotic response element |

## Summary

There is some evidence to suggest that human papilloma virus (HPV) may play a causal role in head and neck carcinoma (HNSCC). The aim of this study was to investigate the prevalence of HPV DNA in HNSCC and to determine whether any correlation exists with p16 or survival.

An initial pilot study of sixty formalin-fixed HNSCC was carried out in order to optimise the methodology for the PCR and immunohistochemistry. A further 84 benign lesions, 12 dysplasias and additional 80 HNSCC were also included.

In the pilot study the prevalence of all HPV types was 67% of which 18% were high risk-HPV (HR-HPV) and for the combined carcinoma sample it was 59% of which 25% were HR-HPV. The overall HPV prevalence was 51% and 42% for benign lesions and dysplasias with HR-HPV accounting for 14% and 8% respectively. A total of four alpha HPV types were identified and eleven beta HPV types. Multiple HPV types co-existed in the same tissue and in some cases both alpha and beta HPV. The results may suggest that HR-HPV may play a role in a small subset of HNSCC. An association was found between HPV status and gender, age group, survival, nodal metastasis and $T_3$ tumour size and smoking. HPV16 was predominantly present in female patients and was associated with an improved overall survival and recurrence free survival.

p16 positivity varied from 76-78% in carcinomas, 51% in benign lesions and 66% in dysplasias. p16 status was not associated with disease recurrence or nodal metastasis.

Positive p16 staining and high staining intensity was associated with a poorer overall survival and the male gender, an older age group, anatomic site, and $T_2$ tumour size.

Overall HPV status was not correlated with p16 expression but a correlation found between p16 and HPV16 may suggest that p16 could potentially act as a surrogate marker of HPV16. However, the lack of concordance would suggest that in isolation p16 may not be a reliable marker for HR-HPV and should not be relied upon in isolation. Our findings could suggest that HPV16 and p16 status may be independent predictors for prognosis and disease recurrence.

# CHAPTER 1

# INTRODUCTION

## 1.1 ORAL CARCINOGENESIS

### 1.1.1 What is cancer?

Cancer is one of the five major causes of mortality across all society. Generally, cancer is a manifestation of a malignant neoplasm or tumour, which is an unusual mass of tissues (except for leukaemias and other cancer types) generated as a result of uncoordinated growth of that tissue. It is distinctively different from a benign neoplasm which is localised and can normally be removed from the affected part of the body. So, cancer is a malignant neoplasm which has the capability to invade neighbouring tissues (invasion) and spread to other parts of the body (metastasise).

Cancer is essentially a chronic disease, usually being classified according to the tissue of origin. To date, more than 150 different types of human cancers have been recognised.

### 1.1.2 Head and neck cancer

Head and neck cancer encompasses epithelial malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity (including the buccal mucosa, upper and lower alveolar ridges, retromolar trigone, floor of the mouth, hard and soft palate and anterior two-

thirds of the tongue), oropharynx, nasopharynx, hypopharynx, pharynx, and larynx (Marur and Forastiere, 2008). The incidence and mortality of head and neck cancer varies throughout the world. Globally, it is the sixth most common cancer type, accounting for 6 % of all cases, and responsible for 650 000 new cancer cases annually with a mortality of 350 000 cases annually worldwide (Argiris et al., 2008).

Gender and racial differences in head and neck cancer incidence and mortality appear to reflect differences in risk factor exposure. As with most cancers, age itself may be a risk factor for the development of head and neck cancer. The epidemiology of head and neck squamous cell carcinoma has changed considerably in this century. Risk factors appear to explain most of the geographic, racial and gender variations seen, as well as incidence changes over time (Davidson, 2001). The history of previous head and neck cancer is one of the best predictors of cancer risk. Previously treated head and neck squamous cell carcinomas patients have a risk of developing second cancers of the head and neck, esophagus and lung (Braakhuis et al., 2003; Ha and Califano, 2003).

The risk of second cancers appears to relate to tobacco and alcohol exposure as was previously believed and described by the concept of field cancerisation (Lydiatt et al., 1998; Mignogna et al., 2007). The progress of this concept will be discussed further in the later part of this chapter in relation to the development of multiple primary lesions. There are variations in risk related to tobacco types and delivery methods and also alcohol consumption. Accurately measuring these risk exposures is complex. Other factors may act in concert with tobacco and alcohol exposure to contribute to the epidemiology of this disease.

### 1.1.3 Oral cancer

As a subset of head and neck malignancy, oral cancer is specifically found in the oral cavity (sites as mentioned in 1.1.2), including the lips, salivary glands and oropharynx (Marur and Forastiere, 2008). The latest version of the International Classification of Diseases (ICD version 10) coding system of the World Health Organization (WHO) categorised these cancer sites as: the lip (ICD-10, C00); base of tongue (ICD-10, C01); other and unspecified parts of tongue (ICD-10, C02); gum (ICD-10, C03); floor of the mouth (ICD-10, C04); palate (ICD-10, C05); other and unspecified parts of the mouth (ICD-10, C06); parotid gland (ICD-10, C07); other and unspecified parts of major glands (ICD-10, C08); oropharynx (ICD-10, C10); nasopharynx (ICD-10, C11); pyriform sinus (ICD-10, C12); hypopharynx (ICD-10, C13); other and ill-defined sites in the lip, oral cavity and pharynx (ICD-10, C14) (Johnson, 2003).

### 1.1.4 Classification of oral cancer

Oral cancer is categorised by specific location and the cell-type of origin. There are two types of oral cancer clearly recognised according to location, i.e. oral cavity and oropharyngeal cancer. Oral cavity cancer includes the tongue, the floor of the mouth, the hard palate, upper and lower jaw, gums and teeth, the cheeks and salivary glands. Oropharyngeal cancer on the other hand, includes the base of the tongue, uvula and soft palate (Morrow, 2007).

Ninety percent of all diagnosed oral cancers are squamous cell carcinoma. The second commonest type is minor salivary gland cancer and, therefore, glandular in origin (Morrow, 2007).

### 1.1.5 Epidemiology of oral cancer

Despite increased understanding of oral cancer carcinogenesis, fully supported by the most recent technology including combined treatment modalities of oral cancer management, diagnosis has not improved significantly with regard to the 5 year overall survival rates for the disease over the past three decades especially in the United Kingdom (UK) (Rapidis et al., 2009; Warnakulasuriya, 2009).

Based on global incidence, the group of oral and pharyngeal cancers rank sixth among human cancers (Warnakulasuriya, 2009). Geographic variations in oral cancer incidence are greatly influenced by factors such as genetic, habits and lifestyle (Moore et al., 2000). High-risk countries for oral cancer are India, Pakistan, Sri Lanka and Bangladesh. Although, Scotland shows higher rates for oral cancer in both genders within the UK, oral cancer incidence in the UK region as a whole shows relatively lower rates (5325 new cases in 2006) compared to other European Union (EU) countries (Warnakulasuriya, 2009). In the UK, 1851 deaths were caused by oral cancer in 2007 and with Scotland showing higher death rates for both sexes (6.4 and 3.3 per 100,000 population for male and female, respectively) than the rest of the UK (Cancer Research UK 2006, Mortality: http://info.cancerresearchuk.org/cancerstats/ types/oral/mortality/).

About 95% of all oral cancer cases occur in people over the age of 50 and twice as frequently in men as in women. Nowadays, a new trend of oral cancer in young people before the age of 40 is emerging and the male:female ratio has been changing significantly, from 5:1 to 2:1, over the last five decades (Cancer Research UK 2006, Incidence: http://info.cancerresearchuk.org/cancerstats/types/oral/incidence/).

In Asian populations, the buccal mucosa is the most common intraoral site for mouth cancer, whereas tongue is the commonest among European and US cases (Warnakulasuriya, 2009).

### 1.1.6 Aetiology of oral cancer

The exact causes of oral cancer remain unknown due to the multifactorial nature of carcinogenesis (Scully et al., 2005). Studies on inherited genes thus far only explain a proportion of all cancers. A genetic predisposition for oral cancer is at best small but does not preclude it being important in a minority of cases. However, the effects are swamped by environmental factors on a population basis (Scully and Boyle, 2005; Shah and Gil, 2009). Nevertheless, determination of the reality and nature of these genetic factors would have enormous benefit, not only to 'at-risk' family members, who would thus take particular care to avoid these risks, but also in unravelling the molecular mechanisms of oral carcinogenesis, opening the way to better prevention and treatment (Rapidis et al., 2009; Scully and Bagán, 2009 and Petersen, 2009).

### 1.1.7 Risk factors for oral cancer

It is widely considered that the major risk factors for oral cancer are tobacco smoke (IARC, 1988; IARC, 2004), alcoholic beverage drinking (IARC, 1988) or synergy between the former and later factors (IARC, 2007).

#### 1.1.7.1 Tobacco smoking

More than 300 carcinogens are present in either tobacco smoke or its water-soluble components in heavy smokers' saliva (IARC, 2004). The first 69 carcinogens were previously reported and categorised into eleven groups as follows: polynuclear aromatic hydrocarbons (PAHs), heterocyclic hydrocarbons, volatile hydrocarbons, nitrohydrocarbons, aromatic amines, *N*-heterocyclic amines, *N*-nitrosamines, aldehydes, miscellaneous organic compounds, inorganic compounds and phenolic compounds (Hoffmann *et al.*, 2001). It was observed that dose-related tobacco smoking practice (i.e. the frequency of cigarettes smoking and duration of smoking) is strongly associated with oral cancer (Rodu and Jansson, 2004; IARC, 2006; Warnakulasuriya et al., 2007 and Weitkunat et al, 2007).

An increase in metabolism of PAH, which is caused by the up-regulation of the CYP1A1 and CYP1B1 (cytochrome P450 related enzymes) and finally leading to the release of diolepoxides (biologically reactive intermediates), can result in DNA damage (Figure 1.1, Penning and Lerman, 2008). In this respect diolepoxides, which are the product of monoxygenation of PAH by both enzymes, form covalent bulky adducts with DNA that may lead to mutation.

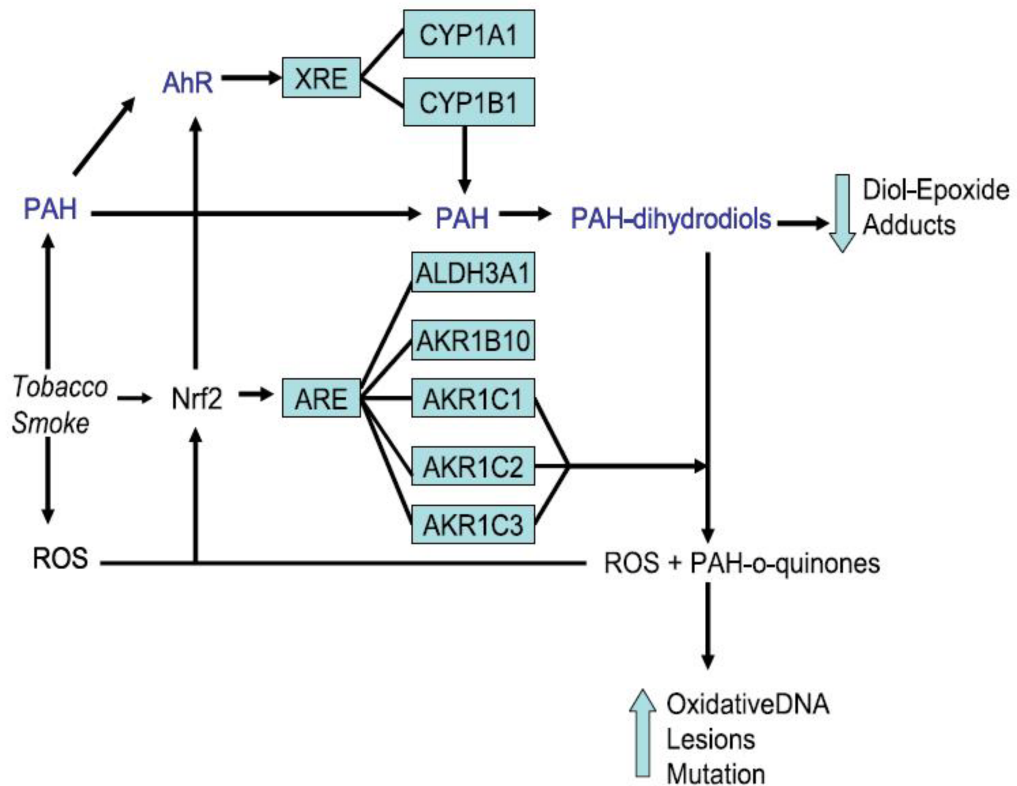



**Figure 1.1: Gene alteration results of tobacco smoking.**
Reprinted with permission from Penning and Lerman (Penning and Lerman, 2008)
AhR, aryl hydrocarbon receptor; AKR, aldo-keto reductase; ALDH, aldehyde dehydrogenase; ARE, antioxidant response element; CYP, cytochrome P450 related enzyme; PAH, polynuclear aromatic hydrocarbons; ROS, reactive oxygen species; Nrf, nuclear respiratory factor2; XRE, xenobiotic response element.

#### 1.1.7.2 Smokeless tobacco

Smokeless tobacco (ST) is an alternative way of consuming tobacco, whereby combustion is not required. Nevertheless, nasal snuff in various forms (loose or small packet), and chewing tobacco (in block or flakes) still keeps mucous membranes in contact with the carcinogenic agent(s). It is commonly practiced in America or Europe, and carries at most a minor increase risk of oral cancer (Rodu and Godshall, 2006).

Rodu and Jansson summarised that cancer-inhibiting agents exist in tobacco leaf (which has cancer protection characteristics) in their review involving case-controlled studies, restricted mainly to the United States and Sweden (Rodu and Jansson, 2004). Carotenoids and phenolic compounds are present in tobacco leaf and have antioxidant properties. Smokeless tobacco use is believed to reduce oral cancer risk. Rodu and Godshall stated that smokeless tobacco use had been associated with oral cancer and contradicts previous epidemiological studies (Rodu and Godshall, 2006). Moreover, elevated cancer risks in specific populations or from specific products cannot be excluded (Weitkunat et al., 2007). Several issues were addressed by Boffetta and colleagues on smokeless tobacco practice including oral use in several regions. Nevertheless this practice could not minimise cancer risks to this group than non-users of any form of tobacco use (Boffetta et al., 2008).

#### 1.1.7.3 Alcohol

The conversion of ethanol to acetaldehyde is catalysed by alcohol dehydrogenases (ADH), and the formation of acetic acid from acetaldehyde is catalysed by aldehyde dehydrogenases (ALDH). Most alcohol and aldehyde metabolism occurs in the liver, but metabolism by ADH, the cytochrome P450–related enzyme CYP2E1, and ALDH is carried out in the upper aerodigestive tract (UADT) (include the oral cavity, pharynx, larynx, and esophagus) (Pöschl and Seitz, 2004). A few studies focused on isoenzymes related to ethanol metabolism, such as ADH (Peters et al., 2005), CYP2E1 (Figaro Gattá et al., 2006) and ALDH with expectation that such enzymes might be found as a convincing risk factor for OSCC (Hashibe et al., 2007; Ho et al., 2007).

It is well known that the active substance in alcoholic beverages is ethanol and regular consumption is directly associated with increased oral cancer risk in a dose-dependent relationship (Petti, 2009). The mutagenic effect of acetyldehyde, a product of ethanol metabolism, in oral cancer is well established in animal research although there is hitherto insufficient evidence to support this in humans (Figuiero-Ruiz et al., 2004). The source of this mutagen is not only alcohol metabolism but also from oral microflora activity, particularly Streptococci species (Kurkivuori et al., 2007).

Tables 1.1 and 1.2 show selected investigations on alcohol as a risk factor in oral cancer (with findings) and a compilation of supporting evidence on either alcohol as an independent risk factor or in combination with other risk factors in oral cancer (in descending order of year of publication), respectively.

**Table 1.1: Selected findings on alcohol with other risk factors in oral cancer**

| **Study (first author, year)** | **Conclusions** |
|---|---|
| Bagán and Scully, 2009 | It was noted that the frequency and duration of tobacco chewing and alcohol drinking and duration of bidi smoking were the manifestation of dose–response relationships. |
| Hennessey, 2009 | Sexual history is more relevant than tobacco or alcohol as the major risk factors for HPV-positive HNSCC, but this is not the case for HPV-negative HNSCC. |
| Hooper et al., 2009 | A recent study has shown that alcohol consumption may also change microflora level in the oral cavity such as *Streptococcus anginosus*. It was reported that this microbes were significantly higher in alcoholics compared with non-alcoholic patients. |
| Lucenteforte, 2009 | It was reported that heavy alcohol drinkers tend to have a limited intake of other more beneficial foods especially essential nutrients. |
| Petersen, 2009 | Although heavy intake of alcoholic beverages is related to nutrient deficiency, study results have shown that both act independently in oral carcinogenesis. |
| Petti, 2009 | Globally, 7-19% of oral cancers are associated with alcohol intake. |
| Scully and Bagán, 2009 | Current trends have demonstrated that there has been a slight increase in alcohol and tobacco use in women. |
| Shah and Gil, 2009 | Even though tobacco and alcohol consumption remain the major etiologic factors, recent findings have shown that HPV has been implicated in oral cancer. |
| Marichalar-Mendia, 2010 | The actual role of alcohol in oral carcinogenesis remains unknown, but regular alcohol consumption might be a risk factor in oral tumorigenesis based on epidemiological studies. It is thought that almost 20% of oral cancer cases are associated with heavy alcohol intake. |

## Table 1.2: Reported evidence for alcohol with others risk factors in oral cancer

| Alcohol only (28 studies) | Alcohol-Smoking (36 studies) | Alcohol-Others (21 studies) |
|---|---|---|
| Cancela et al., 2009 | Mayne et al., 2009 | Muwonge et al., 2008 |
| Massarrat, 2008 | Varshney et al., 2003 | Ide et al., 2008 |
| Salehi et al., 2008 | Adewole, 2002 | Ho et al., 2007 |
| Rodu & Phillips, 2007 | Girja et al., 2002 | Güneri et al., 2005 |
| Farshadpour et al., 2007 | Chen et al., 2001 | Shui & Chen, 2004 |
| Kruk and Aboul-Enein, 2006 | Zavras et al., 2001 | Liao et al., 2003 |
| Petti & Scully, 2005 | Hindle et al., 2000 | Lissowska et al., 2003 |
| Ogden, 2005 | Khan et al., 2000 | Znaori et al., 2003 |
| Figuero Ruiz et al., 2004 | Moreno-Lopez et al., 2000 | Vora, 2000 |
| Pöschl and Seitz, 2004 | Castellsagué et al., 1999 | Rao & Desai, 1998 |
| Salaspuro et al., 2003 | Franceschi et al., 1999 | Takezaki et al., 1996 |
| Das et al., 2003 | Hayes et al., 1999 | Ying-Chin et al., 1995 |
| Huang et al., 2003 | Katoh et al., 1999 | Macfarlane et al., 1995 |
| Yokoyama & Omori, 2003 | Kerawala, 1999 | Kabat et al., 1994 |
| Zavras et al., 2002 | Kurumatani et al., 1999 | Rao et al., 1994 |
| Mignogna et al., 2001 | Warnakulasuriya & Johnson, 1999 | Day et al., 1993 |
| Schwartz et al., 2001 | Talamini et al., 1998 | Kune et al., 1993 |
| Holmes-McNary, 2001 | Canto et al., 1998 | Marshall et al., 1992 |
| Teschke et al., 2001 | Schildt et al., 1998 | Zheng et al., 1990 |
| Cortese et al., 2000 | de Boer et al., 1997 | Elwood et al., 1984 |
| Franceschi et al., 2000 | Sanderson et al., 1997 | Graham et al., 1977 |
| Hindle et al., 2000 | Szabo et al., 1997 | |
| Maier et al., 1999 | Fabian et al., 1996 | |
| Ogden & Wight, 1998 | Bundgaard et al., 1995 | |
| Wight & Ogden, 1998 | Bundgaard et al., 1994 (NSND) | |
| Harty et al., 1997 | Llewelyn & Mitchell, 1994 | |
| Harris, 1997 | Day et al., 1994 | |
| Palmer, 1985 | Mashberg et al., 1993 | |
| | Choi & Kahyo, 1991 | |
| | Herschfus, 1991 (synergy) | |
| | Talamini et al., 1990 (NS/ND) | |
| | Wey et al., 1987 (NSND) | |
| | Kune & McLaughlin, 1983 | |
| | Kuschnir, 1980 | |
| | Johnston & Ballantyne, 1977 | |
| | Vincent & Marchetta, 1963 | |

Abbreviation:- NSND – non-smoking and non-drinking; NS/ND – either non-smoking or non-drinking

#### 1.1.7.4 Synergy between smoking and alcohol

Taking both risk factors together, the total proportional impact of tobacco and alcohol on upper aero-digestive tract (UADT) cancer is around 80%, and yet only a small percentage of affected individuals with these carcinogens in their background develop neoplastic lesions (Canova et al., 2009) (Table 1.2, middle column). In his review on the influence of lifestyles on oral cancer prevalence, Petti provided evidence that the synergistic effect of both factors is more prominent than the other risk factors (Petti, 2009). Despite agreement on the conjoint impact of both factors, looking at them separately for an effect was unlikely to be meaningful in view of their tendency towards confounding characteristics in patient populations (Smith et al., 2007). There is no doubt on the effect of both tobacco smoking and alcohol drinking as evidence by an excessive frequency of second primary cancers (SPCs) observed in HNSCC (Chuang et al., 2008). The prevalence of second primary tumours (SPTs) observed in non-smokers and non-drinkers (NSND) were statistically comparable in both smokers and drinkers, even though their distinctiveness in clinicopathological properties has been established (Farshadpour, 2007).

#### 1.1.7.5 Betel-quid or areca nut (*Areca cathecu*) chewing

Thomas and co-workers have shown that betel quid chewing and smoking are independent risk factors for oral cancer in a large case-control study in Papua New Guinea where betel quid does not contain tobacco (Thomas et al., 2007). Nair and colleagues (1999) revealed that habitual betel quid/tobacco chewers of Indian ethnicity are at risk of developing oral leukoplakia, a potentially malignant lesion.

#### 1.1.7.6 Nutritional status and iron deficiency

It was reported in one study that deficiencies in iron, vitamins A, C and E, zinc and selenium may be implicated in the aetiology of oral cancer. Low intake of fruit and vegetables has been associated with cancer of the mouth, larynx and oesophagus. It was believed that the active anti-oxidant substances that exist in fruit and vegetables act as suppressor agents to control cell growth. Active ingredients mentioned include indoles, flavonoids, isothiocyanates, terpenes, rutin and phenolic antioxidants (Lucenteforte et al., 2009). The fibre content in vegetables was also found to be associated with a reduction in oral cancer risk. A few studies provided evidence that vitamin A deficiency correlated with oral precancerous lesions and subsequent cancer (Zain, 2001; Lucenteforte et al., 2009); Even though beta-carotene (a precursor to vitamin A and a potent antioxidant as well) could be found in some fruits and vegetables, it has not been shown to control premalignant or cancerous oral lesions developing.

#### 1.1.7.7 Bacteria and other microbial infections

There are a large number of studies investigating the possible relationship between microorganisms and oral carcinomas. Numerous mechanisms by which bacteria and yeast may initiate or promote carcinogenesis have been proposed (Hooper et al., 2009). However, conclusive evidence has yet not getting emerged to show such microbes being involved directly or indirectly in oral cancer.

#### 1.1.7.8 Viruses

More recently, another focus has been the small proportion of patients who develop oral cancer without exposure to one or both of the risk factors (tobacco smoking and/or alcohol consumption) (Farshadpour et al., 2007; Dahlstrom et al., 2008). The aetiology of these unusual and rare cases remains unknown. Therefore, it is possible that other factors are operating such as heredity and other environmental factors such as viral infections and/or nutritional deficiencies. Furniss and colleagues conducted a case-control study involving 1034 pharyngeal cancer patients testing for antibodies to common HPV types. They concluded that the increased risk of HPV6 expression was no direct association with smoking and alcohol consumption in head and neck squamous cell carcinoma (Furniss et al., 2009).

It was noted by Kreimer that approximately 25 percent of all cancer types may be aetiologically connected with viruses (Kreimer et al., 2005). Human papillomaviruses (HPVs) have been proven to be important agents in promoting a diverse set of the head and neck cancers. To date, direct involvement of viruses in oral carcinomas is not strongly supported by the data but it is known that the role of HPVs in some part of the carcinogenesis processes in some human neoplasms is around 23.5% (Shillitoe, 2009). The involvement of HPVs in oral carcinogenesis will be discussed further in a subsequent chapter. There are other types of human viruses such as hepatitis C virus, retroviruses (enveloped viruses) and DNA tumour viruses that could also be involved in cancer (Bellon and Nicot, 2008).

Human papillomavirus (Papovaviridae group: icosahedral, non-enveloped particles, 45-55 nm in diameter, containing double-stranded circular DNA) is among the candidates of DNA tumour viruses that include other viruses such as Simian Virus 40, polyomavirus, human adenoviruses, Epstein-Barr virus (EBV), Hepatitis B virus and Human herpesvirus 8 (HHV8).

DNA viruses downregulate the activity of tumour suppressors such as p53 and retinoblastoma (Rb) protein. Many viruses contain genes that block apoptosis or inhibit immune recognition of the infected host cell, which favour cell survival.

### 1.1.8 Clinical and Histopathological Aspects

The term 'oral cancer' is almost synonymous with the term squamous cell carcinoma (SCC) because of the great numerical dominance of SCCs in mouth cancer. Oral cancer is often difficult to identify at its early stages due to its often asymptomatic nature. In addition, the disease usually does not lead to early symptoms or complaints which would force the patient to seek professional advice.

Three main parameters are usually used for comparing and assessing the different forms of treatment outcomes for oral cancer patients. This includes anatomical description accuracy of the lesion site, the record of the treatment regimen instituted and a reliable system used for pre-therapeutic clinical staging of the lesion. The TNM (T, tumor size; N, node involvement; M, presence of distant metastasis) staging system for head and neck cancers (HNCs) is now in its Seventh Edition (AJCC Cancer Staging Handbook, 2010). It is a vital tool for comparing and reporting therapy outcomes and

for inclusion in clinical trials by improving patient stratification. This system is the most widely used for the clinical staging of tumours of the oral cavity. This system was developed by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC) to identify clinically the stage of primary tumors of the oral cavity. As shown in Table 1.3, Stage IV has been revised in the sixth edition by defining subcategories to reflect therapeutic strategies: Stage IVA is assigned to tumours that are locally advanced but surgically resectable; Stage IVB consists of tumours that are locally advanced and surgically unresectable but potentially treatable with chemoradio-therapy; and Stage IVC includes patients with distant metastatic disease that is incurable and therefore only suitable for palliative treatment (Patel and Shah, 2005).

**Table 1.3: TNM stage grouping for oral cancer**

| Stage of tumour | Primary tumour[a] | Regional lymph nodes[b] | Distant metastases[a] |
|---|---|---|---|
| Stage 0 | $T_x$ | $N_x$ | $M_x$ |
| | $T_0$ | | |
| | $T_{is}$ | $N_0$ | $M_0$ |
| Stage I | $T_1$ | $N_0$ | $M_0$ |
| Stage II | $T_2$ | $N_0$ | $M_0$ |
| Stage III | $T_3$ | $N_0$ | $M_0$ |
| | $T_1$ | $N_1$ | $M_0$ |
| | $T_2$ | $N_1$ | $M_0$ |
| | $T_3$ | $N_1$ | $M_0$ |
| Stage IVA | $T_4$ | $N_o$ | $M_0$ |
| | $T_4$ | $N_1$ | $M_0$ |
| | Any T | $N_2$ | $M_0$ |
| Stage IVB | Any T | $N_3$ | $M_0$ |
| Stage IVC | Any T | Any N | $M_1$ |

Adapted from AJCC Cancer Staging Handbook, Seventh Edition (2010), AJCC

Descriptions:

[a] determination done via clinical examination and radiography
[b] determination done via clinical examination only

$T_x$ Primary tumour cannot be assessed
$T_0$ no evidence of primary tumour
$T_{is}$ preinvasive carcinoma (carcinoma in situ)
$T_1$ $\leq$ 2 cm tumour in its greatest dimension
$T_2$ 2 cm $\leq$ tumour size < 4 cm in its greatest dimension
$T_3$ $\geq$ 4 cm tumour in its greatest dimension
$T_{4a}$ *Lip*: tumour invades through cortical bone, inferior alveolar nerve, floor of mouth or skin of face (i.e. chin or nose)
*Oral cavity*: tumour invades through cortical bone, into deep (extrinsic) muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
$T_{4b}$ Tumour involves masticator space, pterygoid plates, or skull base and.or encases internal carotid artery

*According to 2002 AJCC/UICC staging system for cervical lymph node metastasis*

$N_x$ the minimum requirements to access the regional lymph nodes cannot be met
$N_0$ no regional lymph node metastasis
$N_1$ movable ipsilateral lymph nodes present $\leq$ 3 cm
$N_{2A}$ movable single ipsilateral lymph nodes (3 cm < metastasis < 6 cm) in its greatest dimension or multiple ipsilateral or bilateral or contralateral lymph nodes present $\leq$ 6 cm
$N_{2B}$ multiple ipsilateral lymph nodes, none > 6 cm
$N_{2C}$ Metastasis in bilateral or contralateral lymph nodes, none > 6 cm
$N_3$ any palpable lymph nodes > 6 cm in its greatest dimension
$M_x$ the minimum requirements to access the present of distant metastases cannot be met
$M_0$ no evidence of distant metastases
$M_1$ evidence of distant metastases (blood borne)

### 1.1.9 Field Cancerisation versus Clonal Spread/Monoclonal Origin

This multi-step process of carcinogenesis was first suggested by Slaughter et al. (1953), and focused on multiple lesions arising as result of exposure of the oral epithelium to a carcinogen. This concept has been challenged by the clonal spread theory, based on findings that second primary tumours may be found distant from the original one (monoclonal origin model) and supported by genetic analysis (Califano et al., 1996; Bedi et al., 1996 and Partridge et al., 1997; 1998). There have been a number of studies supporting either or both concepts as represented in Table 1.4.

**Table 1.4: Polyclonal origin versus Monoclonal origin**

| Independent abnormalities Field Canserisation-Slaughter's (Polyclonal Origin) | Support both concepts | Clonal Spread (Monoclonal Origin) |
|---|---|---|
| Leedham et al., 2009 | Klinge and Fiebeler, 2009 | Ostby et al., 2006 |
| Parsons, 2008 | Abou-Elhamd and Habib, 2007 | Pateromichelakis et al., 2005 |
| Gonzáles-Moles et al., 2008 | Scholes et al, 1998 | Partridge et al., 2001 |
| Mignogna et al., 2007 | Worsham et al., 1995 | Braakhuis et al., 2002; 2003; 2005 |
| Raimondi et al., 2005 | Hays et al., 1995 | Brieger et al., 2003 |
| Almadori et al., 2004 | | Nagai et al., 2003 |
| Bagán et al., 2004 | | Vriesema et al., 2001 |
| Thomas et al., 2003 | | Tabor et al., 2001 |
| Thomson et al., 2002 | | Cloos et al., 2000 |
| Nagler, 2002 | | Califano et al., 1996; 1999; 2000; 2003 |
| Jang et al., 2001 | | Garcia et al., 1999 |
| Van Rees et al., 2000 | | Bedi et al., 1996 |
| Martins, 2000 | | |
| Bloching et al., 2000 | | |
| Van Oijen et al., 1999; | | |
| Van Oijen and Slootweg, 2000 | | |
| Lydiatt et al., 1998 | | |
| Mitsudomi et al., 1997 | | |
| Ogden et al., 1997 | | |
| Copper et al., 1993 | | |

*Details of 42 studies supporting either or both concepts are given in the references

The risk of developing second cancers of the head and neck, oesophagus, and lung is approximately 4 percent per year when treated patients are followed over time (Benner et al., 1994). Long term exposure to tobacco and alcohol appears to place large areas of the aerodigestive tract mucosa at increased risk of carcinogenesis (Schwart et al., 1994).

### 1.1.10 Prognostic Factors

Prognosis (comes from the Latin word, prognōsis and from Greek, progignōskein, to foreknow: pro-, before; gignōskein, gnō-, to know) is defined as a prediction of the probable outcome of a disease, based on the individual's condition and the usual course of the disease as seen in similar situations and a second definition is patient's chance of recovery from a disease (Bailey, 1998).

Prognosis on its own is a dynamic process and influenced by multiple factors such as time, the disease itself and the planned intervention. The framework for explaining prognostic factors in cancer was proposed and included subject-based classification and clinical-relevance classification (Gospodarowicz and Sullivan, 2001; Gospodarowicz et al., 2001). Three broad and distinct categories were proposed according to subject-based classification and for clinical-relevance classification. The former classification relates those prognostic factors to tumour, patients and to the environment. The latter classification grouped those factors into essential (basic requirement for making treatment decision of cancer type defined by histology of molecular tumour characteristics), additional (required to define the outcome more precisely but not to lead general treatment decision) and new and promising factors

(mainly refers to molecular factors) (Gospodarowicz et al., 2006). Therefore the combination of both classifications can be simplified as the prognostic factors for oral cancer and is affected by the choice of the planned intervention and the outcome of the interest (Figure 1.2).

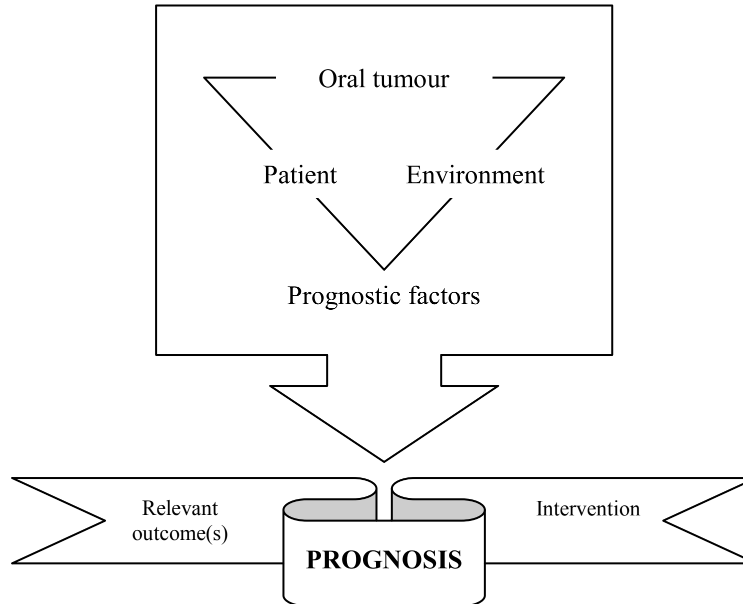



**Figure 1.2: Prognostic factors interaction leading to appropriate prognosis**
(Modified from Gospodarowicz et al., 2006)

Over the years, surgical pathologists and clinicians have sought reproducible and reliable histopathologic factors with possible prognostic implications in oral cancer. The histopathologic factors with potential prognostic significance such as tumour depth, tumour thickness, lymphatic or vascular invasion, perineural invasion, margin of surgical excision, angiogenesis density score, histopathologic scoring and grading system and histopathologic classification of the carcinoma.

Studies on molecular biological markers for predicting malignant transformation of oral pre-malignant lesions have not demonstrated methods that are readily applicable for routine diagnostic work. In future studies, it may be important to evaluate the

combined significance of several markers and/or clinical and histological variables for their prognostic value (Reibel, 2003). Recently, the DNA image cytometry approach in evaluating prognosis for tumour cell ploidy is a promising to supplement to clinical and histopathological parameters and could facilitate malignant and premalignant lesions in HNSCC evaluation (Abou-Elhamd and Habib, 2009).

#### 1.1.10.1 Tumour site

The hard palate, buccal mucosa, gingiva, and tongue were the well known sites for squamous cell carcinoma development. For alcohol drinkers and tobacco smokers, among the site specific intraoral regions to be considered as high-risk were the anterior floor of the mouth and oral tongue (Mashberg and Samit, 1995). Alcohol and other dissolved carcinogens in saliva may remain in prolonged contact with mucosa which most predisposed to develop squamous cell carcinoma. The lower lip is the most common site for oral cancer (Woolgar and Hall, 2009). The anterior two-third of the tongue accounts for 40 percent of oral cancer and is the most frequent site for primary lesions (Woolgar and Hall, 2009). Tongue cancer may spread along the mucosal surface to involve the floor of the mouth and mandible, oropharynx, or deep invasion between muscle fascicles.

In contrast, Shaw and colleagues (2009) concluded that site had less effect on prognosis when comparing 485 consecutive patients with buccal tumours with those of another oral site with reference to prognostic variables. This was supported by another study indicating that site effect was found less certain and might not be considered a significant independent factor within oral cavity (Rogers et al., 2009).

#### 1.1.10.2 Tumour thickness and invasion depth

The thickness of a tumour means a direct micrometer measurement of the vertical bulk of the tumour regardless of the histologic structure of an ulcerative or exophytic form of tumour's growth (Breslow, 1970). Depth of invasion, on the other hand, is the extent of growth into tissues beneath an epithelial surface; where epithelium is destroyed. A few studies have identified tumour thickness as an important prognostic indicator in upper aerodigestive neoplasms, especially as a predictor of regional metastasis (Spiro et al., 1986). However, another study found that the risk of lymph nodes metastasis was not influenced by tumour thickness and depth (Amaral et al., 2004). It was suggested that regional node involvement in OSCC patients could be estimated by tumour thickness as a reliable histopathological parameter for prognosis (Pentenero et al., 2005).

The tumour thickness is among the factors in clinical oncology with greater consensual influence on disease outcome (Massano et al., 2006). Early tongue cancers demonstrate occult spread to the cervical lymph nodes in 20 to 30 percent of cases. A depth of invasion by tongue cancer of greater than 5 mm is associated with an increased incidence of occult metastasis (Woolgar and Hall, 2009). Depth is the most significant predictor of cervical node metastasis in early squamous carcinomas of the oral cavity. Patients with a tumour depth of more than or equal to 5 mm are at an increased risk of harbouring node metastasis, hence should have elective node dissection (Kane et al., 2006). Tumour depth greater than 2 mm is correlated with significantly lower survival and control of disease in the neck. Chen and colleagues believed that invasion depth of more than 3 mm at the time of presentation may be responsible for an increased incidence of neck nodal metastasis (Chen et al., 2008).

1.1.10.3 Tumour staging

Costa et al. (2005) reported the convincing relationship between clinical and histological staging and malignancy scores based on estimation of keratinization degree, nuclear pleomorphism and lymphoplasmocytic infiltrate in OSCC. Therefore, it was suggested that TNM clinical staging might be suitable as a prognostic indicator for OSCC. Other supporting opinion was based on data from a retrospective study that was carried out in 128 patients with SCC of the tongue and floor of the mouth. It was concluded that the conventional histopathological grading system has more prognostic value in oral cancer compared to available biological markers thus far (Weijers et al., 2009). Current studies have suggested the introduction of other parameters to improve existing TNM staging and to enhance more comprehensive dynamic prognosis estimates especially involving disease recurrence and metastasis in head and neck patients (Van der Schroeff and Jong, 2009).

1.1.10.4 Extracapsular spread (ECS)

ECS is defined as extranodal extension of metastatic deposits outside the lymph node capsule it is a noticeably important prognostic factor, associated with higher locoregional recurrence rates, distant metastases, and lower survival rates (Woolgar et al., 2003). Woolgar et al. (2006) found that the 3-year survival probability was similar in those with macroscopic or microscopic ECS (33% and 36%, respectively) and much worse than the rate of 72% for those with strict intranodal metastases. Additionally, it has been found that patients with multiple metastatic nodes have poorer prognosis, and

individuals with multiple nodes with ECS show an extremely short median time interval until disease recurrence as well as higher mortality rates.

#### 1.1.10.5 Perineural invasion

Perineural invasion by squamous cell carcinoma is considered an ominous prognostic sign and had been shown to correlate with an increased incidence of local recurrence, regional lymph node metastasis and a decreased survival (Carter et al., 1985). Rodolico and colleagues demonstrated the association between perineural invasion with the risk of nodal metastasis (Rodolico et al., 2004). Nevertheless, the status of perineural invasion as an independent prognosticator is unclear. A previous study was supported by an observation in tongue cancer that positive perineural invasion was not the only factor to influence the increase of incidence in head and neck metastasis but clearly other related histopathological and clinical parameters at the time of presentation (Chen et al., 2008). Similarly, Jones et al. 2009 suggested that perineural invasion role was not conclusive based on published data to date, but their study demonstrated lymphovascular invasion instead as the potential determining factor in oral cancer patients' survival.

#### 1.1.10.6 Pattern of infiltration

It was noted that the interaction of stromal and cancer cells particularly at the invasive tumour front (ITF) is significantly involved in tumour progression (Bànkfalvi and Piffkò, 2000). The evaluation of ITF as the tumour marker (especially the interaction with intratumoral dendrite cells of squamous cell carcinomas) would be more

meaningful if they could be assessed histopathologically (ITF grading) to indicate survival (Schliephake, 2003). Further reviews have supported that the pattern of infiltration as a result of tumour-infiltrating lymphocytes (TILs) interaction with antigen presenting cells (dendrite) could be of good prognostic value (Bettendorf et al., 2004). A study conducted on tongue cancer demonstrated that infiltration that extended to muscular layer had a higher chance of occult metastasis (Amaral et al., 2004). A review of the clinicopathologic data of 73 patients diagnosed with Stage I–II squamous cell carcinoma of the tongue suggested that depth of infiltration could be beneficial in occult metastasis prediction (Keski-Santti et al., 2007). Despite being sensitive enough to assist in clinical decision-making, its value was hampered by poor specificity.

1.1.10.7 Excision margins

The margin of uninvolved tissue around the excised neoplasm is one of the putative indicators of completeness of surgical removal. The effect of the closeness to a margin and of severe dysplasia at the margin on recurrences and prognosis has not been systematically evaluated. Sieczka et al. (2001) concluded that based on the increase in local recurrence in buccal mucosa cancers that negative margins were not a sufficient estimator of local control. Close surgical margin (the region within 5-mm circumference in OSCC without any evidence tumour at the margin) as an aggressive disease indicator has been considered (Sutton et al., 2003). It was later found that a 5-mm standard practice margin would not be adequate if there was evidence of an infiltrative pattern of spread (Huang et al., 2007) but should include histological assessment especially once the high-risk score observed for the purpose of adjuvant radiation therapy (Brandwein-Gensler et al., 2005). The surgical margins in OSCC resections, involving deep margins

were found as high as 45% to 33% in tumours of the maxillary alveolus, retromolar region, oropharynx and buccal mucosa (Woolgar and Triantafyllou, 2005). It was stated that considering the histological status of resection margin which were disease free has a great influence on prognosis (Massano et al., 2006; Woolgar, 2006).

In addition, the close margin needs to be defined systematically by referring to the width of the margin on outcome to give the best estimate of both local recurrence and survival (Binahmed et al., 2007). Haque et al. (2006) suggested that positive surgical margin after adjustments of related clinical parameters was suitable to indicate higher in mortality. A later study claimed that involved and close margins could be used for mortality predictors (Rogers et al., 2009). Liao et al. (2008) revealed that two independent prognostic factors (pathological margin within 7 mm, pathological tumour for at least 10 mm) were highly associated with disease-specific survival (DSS) and overall survival (OS) for local tumour control. Therefore it was suggested to measure the distance of tumour to nearest deep and mucosal resection margins. It should also be based on the macroscopic and microscopic assessment when assessing the size of an OSCC which usually no obvious invasive front (Woolgar & Triantafyllou, 2009). Proposed classification system for depth of tumor invasion helped to determine the optimal level of surgical resection (to include the buccinator in the clinical classification) and as an important predictor of buccal mucosa SCC prognosis (Ota et al., 2009).

### 1.1.11 Oral precancer role

Conventional clinical and histopathological characteristics are still relevant for the prediction of malignant transformation in oral pre-malignant lesions in routine diagnostic oral pathology (Reibel, 2003). Although premalignant mucosal changes do not always precede oral cancers, such changes warn of risk and present a chance for preventive measures.

#### 1.1.11.1 Dysplasia

Dysplastic lesions can present clinically as white patches (leukoplakias), red patches (erythroplakias), a combination of white and red patches (speckled leukoplakias), erosions or ulcers. The gold standard for a definitive diagnosis of an oral lesion is to perform a biopsy to obtain a histopathological diagnosis due to variable presentation of potentially malignant disorders. The terminology of dysplasia (preneoplastic changes) is a diagnosis defined by the presence of certain histological and cytological atypia (variations in the size and shape of the keratinocytes) but no evidence of invasion (Warnakulasuriya et al., 2008). Generally, the epithelium of precursor lesions may be thick but in the mouth are usually atrophic.

Dysplasia has three grades based on the severity of the architectural disturbance of the epithelium as shown in Figure 1.3. The first grade is *mild dysplasia*, if the disturbance limiting to the lower third of the epithelium with minimal cytological atypia. In addition, the mildly atypical features of the lesions present in the middle third of the epithelium might fall into this group either. In *moderate dysplasia*, on the other

hand, the lesions extend to the middle third of epithelium with a degree of cytological atypia. The third grade, *severe dysplasia*, is when the architectural disorder exists with cytological atypia and starts with greater than two-thirds of the epithelium. The presence of marked atypical even the lesions are not extending into the upper third of the epithelium might also categorise into the last grade (Woolgar and Triantafyllou, 2009).

Significant variation among pathologists based on individual criteria hampered the reliable histopathological diagnosis of oral epithelial dysplasias (Scully et al., 2003). Even though fundamentally accepted two important criteria, architectural features and cytological assessment for grading epithelial dysplasia, unacceptable disagreement of inter- and intra-examiners' interpretation putting patients management with such lesion in dilemma (Warnakulasuriya et al., 2008). The understanding of malignant transformation from dysplastic lesions to oral cancer probably depends on prospective molecular markers (Scully et al., 2003; Arduino et al., 2009; Eversole, 2009; Matta et al., 2009; Ralhan et al., 2009; Torres-Rendon et al., 2009; Tsui et al., 2009) and in combination the former with histopathological data (Pitiyage et al., 2009) as the risk predictors.

The concept of carcinoma *in situ* explains the occurrence of malignant transformation (at viable cellular layer as nearly full thickness architectural abnormalities together with significant cytological atypia) but the absence of invasion (Warnakulasuriya et al., 2008).

### 1.1.12 The clinical presentation of potentially malignant lesions

Clinically, leukoplakia is defined as a raised white patch or plaque of oral mucosa measuring 5-mm or more in diameter, which cannot be scraped off and which cannot be attributed to any other diagnosable disease (Batsakis, 2003). Histologically, hyperkeratosis, acanthosis, and subepithelial and perivascular infiltrate may be seen.

Erythroplakia can be described as a bright red, velvety plaque which cannot be characterised clinically or pathologically as being due to any other condition (Kramer et al., 1978). Histologically, most of these lesions tend to show squamous cell carcinomas or severe dysplasias (Shafer and Waldron, 1975).

### 1.1.13 Potential malignant conditions

Oral lichen planus (OLP) is a benign lesion with a characteristic white, lacy, reticular pattern that classically presents on the buccal mucosa. Papular, atrophic or erosive lesions constitute the major subtypes of OLP, and present infrequently. Erosive lesions, in particular, may be quite painful and result in multiple complications, such as secondary infections (predominantly Candida species), as well as poor nutrition and dehydration because of pain (Katta, 2000). Oral lichen planus lesions are suspected of possessing some cancer potential. It is strongly associated with tobacco use (Bhonsle et al., 1979, Gupta et al., 1980); however, malignant transformation rates (Murti et al., 1986) and the relative risk were not significant enough to confirm malignant potential.

Oral submucous fibrosis (OSF) is a unique chronic disease seen almost exclusively in adult patients from south Asia, where its occurrence has a significant association with areca nut use, principally in betel quid (combined areca nut, betel leaf, tobacco and slaked lime) chewing (Sinor et al, 1990; Dave et al, 1992; Jeng et al, 2001).

## 1.2 HUMAN PAPILLOMAVIRUS

### 1.2.1 General information of HPV

HPVs are non-enveloped DNA viruses about 55 nm in diameter, containing a single molecule of circular double-stranded DNA (Muñoz et al., 2006). Their genome size ranges from 7600 to nearly 8000 base pairs in length in the nucleohistone core with eight early genes (E1 to E8) and two late genes (L1 and L2) encoded (Letian and Tianyu, 2010). These genes are previously transcribed as polycistronic mRNAs (Hebner and Laimins, 2006). An upstream regulatory region (URR) or a non-coding regulatory region called the long control region (LCR), is estimated 0.4-0.7 kb in length, contains the origin of replication, promoters, binding sites for core transcription factors, enhancer and repressor proteins (Chow et al., 2010) (Figure 1.3). The functions of the various HPV proteins are as shown in Table 1.5.

### 1.2.2 Types of HPV

Currently, more than 120 different HPV types have been isolated and identified by various molecular techniques and can be classified according to similarity in DNA sequences or based on risk (Chow et al., 2010). When a comparison of genomic DNA sequence and the biology of these viruses is made, if the sequence homology with respect to existing types is <90%, the HPV is classified as a new type; if the homology is 90-98% it is classified as a sub-type; if homology is ≥ 98% it is classified as a variant (Doorbar, 2005).

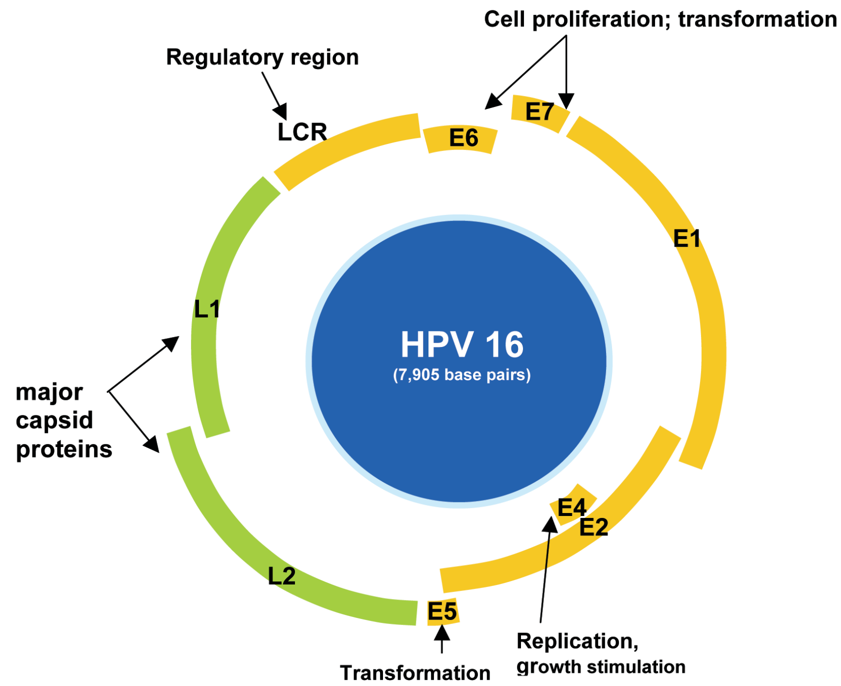



**Figure 1.3: Genome map of HPV16**
Reprinted with permission from Villa (Villa, 2006).

**Table 1.5: The main role of HPV proteins**

| Protein | Roles | Reference |
|---|---|---|
| E1 | Directs initiation of DNA replication<br>Episomal maintenance | Ustav et al., 1991;<br>Sarver et al., 1984 |
| E2 | A transcription activator that has auxiliary role in replication | Ham et al., 1991 |
| | Controlling DNA replication and transcription (ORFs E6-E7) | McBride et al., 1991 |
| E3 | *Remains unknown/Not still clear* | |
| E4 | Disrupts cytokeratins and is important for viral release | Doorbar et al., 1991 |
| | Disrupting the cytoskeleton | Roberts et al., 1993 |
| E5 | A cellular membrane protein that interacts with growth factor receptors<br>Interacting with cellular proteins (EGFR) | Petti et al., 1991; 1994 |
| E6 | A transforming protein that targets p53 for degradation by the ubiquitin pathway | Ciechanover, 1994;<br>Hochstrasser, 1995 |
| E7 | A transforming protein that binds to Rb1, p107, p130 and cyclin AICDK2 proteins | Ciccolini et al., 1994;<br>Davies et al., 1993;<br>Tommasino et al., 1993;<br>McIntyre et al., 1996 |
| E8 | *Remains unknown/Not still clear* | |
| L1 | Major capsid (viral coat protein) | Baker et al., 1991;<br>Hagensee et al., 1994 |
| L2 | Minor capsid that associates with genomic DNA (viral coat protein) | Baker et al., 1991 |

**Table 1.6: Human HPV supergroups and selected candidates**

| HPV supergroup | HPV types | Origin of cloned genome (or comments) |
|---|---|---|
| A<br>(Alpha papillomaviruses) | HPV2, 26, 27 and 29 | Verruca vulgaris |
| | HPV3, 10 and 28 | Verruca plana |
| | HPV6 and 54 | Condyloma acuminatum |
| | HPV7 | Butcher's warts |
| | HPV11 | Laryngeal papilloma |
| | HPV13 and 32 | Focal epithelia hyperplasia |
| | HPV16, 18, 33, 35 and 66 | Cervical carcinoma |
| | HPV30 | Laryngeal carcinoma |
| | HPV34 | Bowen's disease |
| | HPV39 and 40 | Penile intraepithelial neoplasia (PIN) |
| | HPV42 and 70 | Vulval papilloma |
| | HPV43 | Vulval hyperplasia |
| | HPV44 | Vulval condyloma |
| | HPV45, 51, 52, 58 and 69 | Cervical intraepithelial neoplasia (CIN) |
| | HPV53 | Normal cervical mucosa |
| | HPV55 | Bowenoid papulosis |
| | HPV56 | CIN, cervical carcinoma |
| | HPV57 | Inverted papilloma of the maxillary sinus |
| | HPV59 and 68 | Vulvar intraepithelial neoplasia (VIN) |
| | HPV61, 62, 64, 67 and 71 | Vaginal intraepithelial neoplasia (VAIN) |
| | HPV72 and 73 | Oral lesion |
| | HPV74 | Cervical lesion |
| | HPV77 | Skin wart |
| | HPV78, 94 | More frequently cause cutaneous than mucosal lesions |
| | HPV81, 83, 84, 86, 87, 89, candHPV85 and candHPV90 | Mucosal lesions |
| | HPV82 | Mucosal lesions, also in benign lesion |
| | candHPV91 | Mucosal and cutaneous lesions |
| B<br>(Beta papillomaviruses) | HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25 and 47 | Epidermodysplasia verruciformis (EV) lesion |
| | HPV36 | Actinic keratosis |
| | HPV37 | Keratoacanthoma |
| | HPV38 | Malignant melanoma |
| | HPV49 | Verruca plana |
| | HPV75 and 76 | Skin wart |
| | HPV80 and 93 | Cutaneous lesions |
| | candHPV92 and candHPV96 | Pre- and malignant cutaneous lesions |
| G<br>(Gamma papillomaviruses) | HPV4 | Verruca vulgaris |
| | HPV48, 88 and 95 | Cutaneous squamous cell carcinoma |
| | HPV50 | EV lesion |
| | HPV60 | Epidermoid cyst |
| | HPV65 | Pigmented wart |
| M<br>(Mu papillomaviruses) | HPV1 | Verrucas plantaris |
| | HPV63 | Myrmecia |
| N<br>(Nu papillomaviruses) | HPV41 | Disseminated warts |

Modified from Syrjänen and Syrjänen, 1999 and IACR, 2007

Three major groups are identified as supergroup A (Alpha papillomavirus), supergroup B (Beta papillomavirus) and the remaining group of HPVs are members of supergroup E (Mu and Nu-papillomaviruses) (Syrjänen and Syrjänen, 2005). Table 1.6 illustrates the selected human HPV types for each major supergroup and their infection sites in the body. The alpha papillomavirus group, mucosotropic HPVs are further divided into two classes according to their risk to humans i.e. HR-HPV types (potentially oncogenic) and “low risk”-HPV types (non-oncogenic) (Chow et al., 2010) as shown in Table 1.7.

**Table 1.7: Epidemiologic classification of HPV types in alpha HPV supergroup**

| Risk (candidates) | HPV types |
|---|---|
| High (24) | HPV16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82 and candHPV85 |
| Low (30) | HPV2, 3, 6, 7, 10, 11, 13, 28, 29, 32, 40, 42, 43, 44, 54, 61, candHPV62, 71, 72, 74, 78, 81, 83, 84, candHPV86, candHPV87, candHPV89, candHPV90 candHPV91 and 94 |
| Undetermined (6) | HPV2a, 27, 55, 57, 62 and 77 |

Modified from Muñoz et al., 2006; Campisi et al., 2007, IACR, 2007 and Chow et al., 2010

### 1.2.3 HPV oncoproteins

The E6 and E7 are the critical molecules in viral replication and both are the major mediators of carcinogenesis for high risk types. The E6 and E7 ORF encode small proteins of approximately 150 and 100 amino acids, respectively. Both proteins contain C-x-x-C motifs (four for the former and two for the latter) which cause transcription activation, transformation, immortalisation and the association with cellular proteins. The E6 COOH-terminal region contains a post synaptic density protein (PSD95),

drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) (PDZ) domain for interaction with several PDZ domain-containing proteins. The E7 proteins incorporate three conserved regions, CR1 ($NH_2$-terminal), CR2 region and CR3 (COOH-terminal). The CR2 region contains LxCxE (for pRB binding) and a casein kinase II phosphorylation site (CKII) (Hebner and Laimins, 2006). E5 ORF encodes 7.0 kDa small proteins of approximately 44 amino acids which function as a transmembrane domain, with a hydrophobic (30 amino acid) N-terminal and a hydrophilic (14 amino acid) C-terminal (Sparkowski et al., 1996).

### 1.2.4 Pathogenesis of HPV infection

It has been established that HPVs are exclusively epitheliotropic, meaning that their infection is specifically localised in epithelial cells of the host. In order to complete their life cycle, they rely on epithelial differentiation (Doorbar, 2005). HPV infection involves highly complex mechanisms which are still poorly understood. For simplicity, the sequences of the commonly shared stages of general viral pathogenesis are adopted here. The chronological events of the HPV life cycle, regardless of genotype could be divided into five distinct phases: infection and uncoating, genome maintenance, the proliferative-phase, genome amplification and virus synthesis.

It is currently believed that the basal keratinocytes of the squamous epithelium become the prime target for HPV infectious particles as this region has a link to the external environment (Syrjänen and Syrjänen, 2005). In respect to anatomic site, skin, oral cavity, upper gastrointestinal tract, larynx, conjunctiva of the eye, genital tract and anal canal could be the viral portals to permit an initiation of HPV infection into the

host which is composed of adsorption, penetration and uncoating of the viral genome. To ensure the successful of initial infection, a break in the stratified epithelium is a prerequisite since HPV is unlikely to enter into an intact squamous epithelium (Egawa, 2003; Syrjänen and Syrjänen, 2005). Thus, it was suggested that either micro trauma had been established prior to infection or targeting of immature squamous epithelium (prone to infection of high-risk mucosal HPV 16) (Doorbar, 2005; Syrjänen and Syrjänen, 2005). In cutaneous skin, on the other hand, hair follicles seem a 'special and important route' for the B1 supergroup of HPV entry (Boxman et al., 2001).

To date, the nature of cell surface receptors to establish initial viral attachment for the HPV-host cell interaction remains controversial and needs to be elucidated. Two putative cellular receptors, the α4β6 heterodimer of integrin and/or the syndecan-1 of heparan sulphate proteoglycans (HSPGs) were suggested to play a crucial role in the binding of HPVs to the cell surface (Letian and Tianyu, 2010). It was believed that endocytosis of clathrin coated vesicles was commonly used by viruses to penetrate the plasma membrane (Culp and Christensen, 2004; Syrjänen and Syrjänen, 2005). Thus far, the release of viral particles from endosomes and their content into the cytoplasm is called uncoating, but is poorly understood. Two other putative alternative uptake pathways have been proposed, caveolar endocytosis and a clathrin- and caveolae-independent pathway, but they remain to be validated by convincing experimental evidence (Letian and Tianyu, 2010). In principle, the mechanism involves capsid transportation, DNA virus release and disintegration of the empty shell in the nucleus which was adopted from picornaviruses (Cann, 1997).

As previously stated the initial replication of HPVs occurs within the basal (and suprabasal) cells of the squamous epithelium. Upon uncoating viral E1 and E2 proteins are expressed to permit an episomal form of the viral genome to be maintained (Wilson et al., 2002) and also as a low copy number in the basal cells of the epithelium (Doorbar, 2005). At this point, normal terminal differentiation is retarded in which suprabasal cell layers are supposed to exit the cell cycle by the action of both E6 and E7 proteins. During productive infection, E7 stimulates cell proliferation by associating with pRB and its normal induction effect towards apoptosis in response to unscheduled S-phase entry is thought to be prevented by E6 action (Doorbar, 2005). Therefore epithelium infected with HPV 16 undergoes cell cycle progression (Sherman et al., 1997; Stacey et al., 2000). The E5 protein, which has hydrophobic properties, could play a vital role in inhibiting death receptor signalling and to overcome the elimination of infected cells during the early stages of HPV infection (Garnett and Duerksen-Hughes, 2006).

Papillomavirus genome amplification occurs in the mid or upper epithelial layers prior to packaging the DNA into infectious particles (Doorbar, 2005). Promoter P3, which resides within the E7 gene, was thought to be up-regulated leading to the increased expression of E1, E2, E4 and E5 proteins which are responsible for DNA replication (Doorbar, 2005, Chow et al., 2010).

The final phase of HPV infection involves the undistinguishable steps: assembly, maturation and release (Cann, 1997). The expression of two structural proteins, L2 (a minor coat protein) first followed by L1 (a major capsid protein) permits assembly in the upper layers of the infected tissue (Florin et al., 2002). As HPV are non-lytic, it was

believed that by limiting its epitopes/antigen presentation strategy this may prevent it from being detected by the host immune machinery until the cell reaches the uppermost epithelial layers (Matthews et al., 2003).

### 1.2.5 Detection and identification of HPVs

One of the historical dates in HPV research was 1983, when Syrjänen et al. (1983) first proposed HPV's involvement in head and neck carcinogenesis (Syrjänen et al., 1983). Since then, we have seen the rapid development of various detection methods with the vast majority of them focusing on HPV's prime target, keratinocytes. A few controversial issues still exist in the details of the mechanism involved in HPV-host interaction. There is no doubt that the holistic understanding of the significance of the role of HPV in oral carcinogenesis might be exploited for diagnostic, prognostic and treatment purposes.

Clearly, keratinocytes infected with HPV exhibit structural and functional changes in comparison to normal cells (Syrjänen and Syrjänen, 2005). Two distinct types of infections can be outlined, a productive infection and a transforming infection. The former could result in benign epithelial proliferation and normally are detected via microscopic observation of the presence of koilocytosis (Zur Hausen, 1996). The latter could end up with the progression of cancer precursors and invasive cancer. The detection method for this type of infection seems not to be straight forward due to the fact that the cytopathic changes of transformed cells commonly disappear with the progression of cancer. DNA detection techniques could be suggested as an alternative, since morphological means for demonstrating the presence of HPV are generally

unreliable (Syrjänen and Syrjänen, 2005). The percentage of HPV detected in various types of tissue is shown in Table 1.8. (normal), Table 1.9 (benign), Table 1.10 (dysplasia) and Table 1.11 (carcinoma).

#### 1.2.5.1 HPV in cervical specimens

A large number of studies have successfully detected the presence of HPV in cervical tissues. Unfortunately, the reported detection rate (percentage) amongst them and identified HPV genotypes vary due to differences in the HPV detection and genotyping methods. In addition, great variation exists in the sample size of each study. Table 1.12 compares those thirty-five studies from 2007 to 2009, focusing on the sensitivity and the specificity of the detection methods mainly in identifying HR-HPV in the specimens. Out of them, sixteen studies applied two or more different HPV identification methods purposely to review the degree of concordance between or among techniques utilised. It was found the percentage of agreement varied from 33% to 100%.

#### 1.2.5.2 HPV in head and neck clinical samples including oral sites

A Scopus database search for publications from 2000 to 2010 was carried out by using preset keywords: head and neck cancer, oral cancer, HPV identification, HPV genotyping and molecular biology techniques. This ended up with eight relevant studies based on the restricted criteria given above. Similarly, Table 1.13 shows a comparison of parameters as in 1.2.5 (a) among those findings in head and neck clinical samples.

**Table 1.8: HPV prevalence among studies (2000–2010) in normal individuals**

| No. | Investigators | N | Type of tissue | HPV (%) | HPV types |
|---|---|---|---|---|---|
| 1. | Bouda et al., 2000 | 16 | Oral | 0 | - |
| 2. | Patiman et al., 2001 | 7 | Oral | 1/7 (14) | HPV16 |
| 3. | Jimenez et al., 2001 | 20 | Oral | 2/20 (10) | HPV6, HPV16 |
| 4. | Fukui et al., 2001 | 14 | Head and neck | 0 | - |
| 5. | Sugiyama et al., 2003 | 44 | Oral | 16/44 (36) | HPV16 |
| 6. | Kansky et al., 2003 | 66 | Oral | 4/61 (6.6) | HPV11, HPV16, HPV31, HPV68 |
| 7. | ÓFlatharta et al., 2003 | 20 | Oral | 0 | HPV16 |
| 8. | Chang et al., 2003 | 30 | Oral | 6/30 (20) | 17% 'high risk' HPVs |
| 9. | Zhang et al., 2004 | 44 | Oral | 22/44 (55) | HPV16, HPV18 |
| 10. | Kurose et al ., 2004 | 662 | Oral | 4/662 (0.6) | HPV16, HPV53, HPV71, HPV12 |
| 11. | Koppikar et al., 2005 | 102 | Head and neck | 5/102 (5) | n/a |
| 12. | Hormia et al., 2005 | 31 | Gingival | 8/31 (26) | High-risk HPV |
| 13. | Giovannelli et al., 2006 | 17 | Oral | 7/17 (41) | HPV18, HPV16, HPV6, HPV33, HPV53 |
| 14. | Marais et al., 2006 | 116 | Oral | 4/116 (3.5) | HPV13, HPV32 |
| 15. | Kansky et al., 2006 | 45 | Oral | 3/45 (7) | HPV6, HPV11, HPV31 |
| 16. | Luo et al., 2007 | 90 | Oral | 8/90 (9) | High-risk HPV |
| 17. | Gonzalez et al., 2007 | 60 | Oral | 0 | - |
| 18. | González-Losa et al., 2008 | 77 | Oral | 1/77(1.2) | High-risk HPV |
| 19. | Llamas-Martínez et al., 2008 | 30 | Oral | 7/30 (23) | n/a |
| 20. | Lohavanichbutr et al., 2009 | 35 | Oropharyngeal | 2/35 (5.7) | n/a |

**Table 1.9: HPV prevalence among studies (2000–2010) in individuals with oral benign**

| No. | Investigators | N | Type of tissue | HPV (%) | HPV types |
|---|---|---|---|---|---|
| 1. | Jimenez et al., 2001 | 40 | Oral | 22/40 (55) | HPV6, HPV13, HPV32, HPV16 |
| 2. | ÓFlatharta et al., 2003 | 38 | Oral lichen planus | 10/38 (26) | HPV16 |
| 3. | Gonzalez et al., 2007 | 11 | Oral | 10/11 (91) | 30% 'high risk' HPVs |
| 4. | Llamas-Martínez et al., 2008 | 35 | Oral | 16/35 (46) | 40% HPV16 |

**Table 1.10: HPV prevalence among studies (2000–2010) in individuals with oral dysplastic**

| No. | Investigators | N | Type of tissue | HPV (%) | HPV types |
|---|---|---|---|---|---|
| 1. | Patiman et al., 2001 | 30 | Oral | 20/30 (67) | HPV16 |
| 2. | Sugiyama et al., 2003 | 51 | Oral | 31/51 (61) | HPV16 |

**Table 1.11: HPV prevalence among studies (2000–2010) in individuals with carcinoma**

| No. | Investigators | N | Type of tissue | HPV (%) | HPV types |
|---|---|---|---|---|---|
| 1. | Cao et al., 2000 | 40 | Oral | 29/40 (73) | HPV16, HPV18 |
| 2. | Patiman et al., 2001 | 38 | Oral | 35/38 (92) | HPV16 |
| 3. | Fukui et al., 2001 | | Head and neck | 25/98 (26) | HPV16 |
| 4. | Sugiyama et al., 2003 | 86 | Oral | 30/86 (35) | HPV16, HPV18 |
| 5. | Kansky et al., 2003 | 59 | Oral | 5/59 (8) | HPV16, HPV33, HPV58 |
| 6. | Chang et al., 2003 | 103 | Oral | 51/103 (50) | 42% 'high risk' HPVs |
| 7. | Zhang et al., 2004 | 73 | | 54/73 (74) | HPV16, HPV18 |
| 8. | Koppikar et al., 2005 | 102 | Head and neck | 32/102 (31) | 6% HPV16, HPV18 |
| 9. | Giovannelli et al., 2006 | 17 | Oral | 6/17 (35) | |
| 10. | Kansky et al., 2006 | 44 | Oral | 4/44 (9) | HPV6, HPV16 |
| 11. | Luo et al., 2007 | 51 | Oral | 11/51 (22) | High-risk HPV |
| 12. | Gonzalez et al., 2007 | 25 | Oral | 15/25 (60) | 86% 'high risk' HPVs |
| 13. | Llamas-Martínez et al., 2008 | 33 | Oral | 13/33 (39) | 33% HPV16 |
| 14. | Lohavanichbutr et al., 2009 | 119 | Oropharyngeal | 41/119 (35) | n/a |

**Table 1.12: Comparison among various studies for HPV detection in cervical specimens**

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 1 | Chacón et al., 2007 | 272 | PCR HybridCapture Microarray | 33% HPV 16/18 in 212 patients 25% with mixed infection | HPV 16/18 were detected in 55.73% of the 61 patients with H-SIL and cancer, whereas these genotypes were detected in only 7.9% and 22% of women with ASCUS and L-SIL Genotypes 16 and or 18 were detected in most patients with a diagnosis of H-SIL. Other high-risk-HPV genotypes were much less prevalent. Hybrid capture testing is a useful screening test. PCR was effective for identifying genotypes 16 and 18. Histological and cytological findings in cervical samples should be interpreted together with high-risk HPV detection. |
| 2 | He et al., 2007 | 120 cervical swabs | Multi-fluorescent quantitative PCR HybridCapture2 | 52.5% (63/120) positive for HPV | The multi-fluorescent quantitative PCR assay is a simple, quick, sensitive and specific method for genotyping and quantization of HPV infections, it provides strong and detailed data for clinical screening on cervical specimens, virus-load on level of infection and objective mediod on result of HPV infection therapy. |
| 4 | Lee et al., 2007 | 2358 | HPV DNA chip analysis | hr HPV in 23.5% of 1650 normal samples 81.8% of 708 samples with CIN and carcinoma | The major prevalent high-risk HPV genotypes in 381 samples of CIN II/III were HPV-16, -58, -33, and -31, in order of prevalence rate (average overall, 78.0%), and HPV-16, -18, -58, and -33 (average overall, 81.2%) in 133 samples of squamous cell carcinoma (SCC). HPV DNA chip analysis may be a reliable diagnostic tool for the detection of cervical neoplasia and that there are geographic differences in the distribution of high-risk HPV genotypes. |
| 5 | Lin et al., 2007 | 8900 and 7 extrinsic controls | EasyChip® HPV blot | 1–50 copies of HPV genome equivalent (overall) | There was no cross-reactivity with amplicons of other HPV genotypes. The intra-batch and inter-batch reproducibility was 98 and 97%, respectively. The EasyChip® HPV blot is a highly sensitive, reliable and reproducible tool for detection and identification of HPV genotypes. |
| 6 | Park et al., 2007 | 60 (LSIL (30) and LSIL-MA (30)) | $SPF_{10}$ PCR and line probe assay | n/a | H-SIL on follow-up was significantly more common in patients with LSIL-MA (36%) than in patients with LSIL (7%), and negative follow-up was significantly more common in patients with LSIL (50%) than LSIL-MA (23%). |
| 7 | Campos et al., 2008 | 57 | PCR; Roche Linear Array HybridCapture2 | 56/57 (98%) HPV detected | In that study, concordance between the two detection techniques was good and the authors concluded that the medium adequately preserves HPV DNA. The present study adds further knowledge on the compatibility of conventional DNA extraction with the automated Linear Array amplification procedure. |
| 8 | Castle et al., 2008 | 5659 (1427 for LA) | $SPF_{10}$ LiPA Linear Array HybridCapture2 | 83.3% (overall agreement) and 86.9% (agreement among specimens) for HPV positive by both methods. | Both HPV genotyping methods showed excellent agreement for common HPV genotypes detected in baseline cervical specimens. The use of both methods will provide an accurate estimate for the main outcomes in HPV vaccine trials, and both will be useful in studies of the natural history of HPV. |

Abbreviations: CIN-cervical intraepithelial neoplasia; ASCUS-atypical squamous cells of undetermined significance (ASCUS); L-SIL and H-SIL-lowgrade and high-grade squamous intraepithelial lesion; MA-marked cytological atypia

**Table 1.12: (continued)**

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 9 | Jeronimo et al., 2008 | 1745 | Roche Linear Array | 1018/1745 (58.3%) positive for HPVs | Standardization of HPV typing is worth the effort because it forms the basis for HPV research and might play a central role because of its role in defining the persistence of HPV, in clinical decision making for patient management, and even in treatment. |
| 10 | LaMere et al., 2008 | 572 (143 x 4) frozen cervical specimens | PCR, multiplex hybridisation by Luminex® xMAP® | Tested: no HPV (6%), one HPV (62%), ≥ 2 HPVs (32%) genotypes Controls: no HPV (8%), one HPV (63%), ≥ 2 HPVs (29%) genotypes | There was no difference by group. HPV viral DNA was resilient for up to 6-months of frozen storage in the typical alkaline conditions employed for denaturing DNA for hc2 testing. Hybrid Capture 2-tested specimens stored under these conditions can be HPV genotyped using this PCR-based genotyping method. |
| 11 | Lin et al., 2008 | 433 cervical swabs | EasyChip® HPV blot (modified) vs L1-type specific | 419/433 (96.8%) - HPV positivity; 394/433 (91.0%) - type-specific HPV | The modified MY11/GP6+ PCR-based HPV Blot assay is generally satisfactory for clinical HPV genotyping in cervical swab samples. |
| 12 | Mo et al., 2008 | 470 | Hybrid Capture-II® vs AMPLICOR®, INNO-LiPA | 241/470 (51.3%) both positive; For HR HPV, both similar in sensitivity (96.4%) and specificity (100%). | Both AMPLICOR® HPV Test and HCII® serve as excellent systems with similar efficacy for the detection of HR-HPV DNA. The AMPLICOR® HPV Test is efficient, sensitive and can be used routinely to evaluate HPV status, especially in women with an ASC-US diagnosis. |
| 13 | Nazarenko et al., 2008 | 122 cervical samples; 603 (450 +HC2 & 153 -HC2) STM samples | PCR, Hybrid Capture2, Luminex® XMAP® | 115/122 (94.3%); 434/450 (96.4%) +HC2/+PCR; 143/153 (93.5%) -HC2/-PCR | This new sequence-specific Hybrid Capture® sample preparation is fast, efficient and allows direct HPV genotyping by PCR and slightly better detection of multiple HPV infections. |
| 14 | Nielsen et al., 2008 | 10,544: aged 20-29; 1443: aged 40-50; women | Hybrid Capture2, $SPF_{10}$-LiPA | HR HPV prevalence was 17.9% (aged 20-29 years) and 4.4% (aged 40-50 years) | HR HPV infection was common among younger women, with HPV16 as the predominant type. Sexual activity is important for the risk of HR HPV infection and the role of sexual behaviour for the risk of having multiple HR HPV types. |
| 15 | Nobre et al., 2008 | 27: HPV positive patient samples 30: cervical samples | *In silico* analysis PCR-RFLP | HPV DNA positive in 16/30 (53%) (3 normal, 3 low grade-, 5 high grade-lesions and 5 carcinoma) | This approach offers significant advantages over the PCR-RFLP techniques, and other currently available HPV typing assays. It particularly suited for routine HPV detection and identification in settings of poor financial resources, like cervical screening programs in developing countries. |
| 16 | Sabol et al., 2008 | 153 (126 HSILs, 27 LSILs) samples | Linear Array vs INNO-LiPA vs non-commercial (GP-RLB) assay | HPV types were detected by LA, LiPA, GP-RLB, and BS-RLB assays, in 94.1%, 92.8%, 88.2% and 94.1%. | By LA, LiPA, GP-RLB, and BS-RLB assays, the multiple infections were detected: 55.6%, 37.3%, 43.1%, and 52.9% samples, respectively. The majority of double infections: 58.8%, 54.4%, 66.7%, and 60.5% by the LA, LiPA, GP-RLB and the BS-RLB assay, respectively. A large variability in the ability of a particular assay to detect different HPV types. The LA and BS-RLB assays found larger numbers of cases positive for multiple types than the two other assays did. The lowest capability of detecting multiple infections was observed for LiPA. The interassay agreement was moderate for single infections and poor for multiple infections. |

Abbreviations: STM-specimen transport medium; n/a-not available

## Table 1.12: (continued)

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 17 | de Antonio et al., 2008 | 218 | HybridCaptureII® Linear Arrays | For HR HPV detection, +HC2/+LA: 94/218 (43.1%); -HC2/-LA: 105/218 (48.1%) | The two tests used in this study for the detection of HPV in cervical samples – hybrid capture and linear array – useful and reliable tests to detect the presence of HPV cervical infection. |
| 18 | Baleriola et al., 2008 | 834 | HGS High-Risk HPV detection kit; HybridCaptureII® | Specificity: HGS and HCII tests were 90.6% and 84.6%, Sensitivity: HGS and HCII tests were 63.1% and 64.7% | HGS test is simpler, less sample volume, less time and does not require as much specialized equipment (e.g. Luminometer) viable and more accurate alternative to the Hybrid Capture II Assay. |
| 19 | Schmitt et al., 2008 | 1085 | PCR and hybridisation by Luminex | 1085 samples and 27 HPV types analysed (total=29295), 639 (2.2%) +ve concordant, 28,378 (96.9%) -ve concordant and 278 (0.9%) discordant | BSGP5+/6+ multiplexed with β-globin PCR provides an improvement in type-specific amplification sensitivity and homogeneity compared to GP5+/6+ and offers simultaneous internal control of DNA quality. BSGP5+/6+-MPG, therefore, is suitable for epidemiologic and also diagnostic applications. |
| 20 | Stevens et al., 2008 | 142 cervical brush | BeeBlot vs Linear Array | 100/142 (70.4%) HPV detected. The HPV genotyping profiles obtained were identical using either the manual or automated procedure (concordance of 100%) | The BeeBlot automated platform, as a supplementary tool with the LA-HPV test, has a capacity equal in sensitivity to the current recommended detection protocol for typing single and multiple HPV infections. The BeeBlot automated hybridisation and detection system could quite effectively be utilized for processing LA-HPV strips upon appropriate internal laboratory validation. |
| 21 | Brandao et al., 2009 | 147 (51 were pregnant and HIV +ve, 51 HIV +ve and not pregnant and 45 pregnant and HIV-ve) | PCR HybridCapture | HPV positive was 122/143 (85.3%). Among HIV-positive pregnant women: HPV prevalence of 48/50 (96%), of whom 29/48 (60.4%) were high-risk. | A high prevalence of HPV infection was identified, especially with the high-risk types 16, 58, 18 and 66. This study identified high-risk HPV types in all three groups examined (HIV-positive pregnant women, HIV-negative pregnant women and HIV-positive not pregnant), characterising its distribution in this setting. |
| 22 | Brebi et al., 2009 | 41 cervical biopsies of adenocarcinoma | PCR and Reverse Line Blot | HPV positive was (29/41) 71%; HPV 16 (61%); HPV 18 (19.5%) | Eighty seven percent of biopsies had a single HPV infection. Three patients had a multiple HPV infection. A high prevelence of HPV 16 and a low prevalence of HPV 18, which historically has been related to adenocarcinoma. Technique. |
| 23 | Brismar et al., 2009 | 84 CIN with cone biopsies; ectocervix | PCR and Roche Linear Array HPV genotyping | HPV was detected in 65/84 (77%) cone specimens. The most common high-risk types in the cones were HPV 16, 18, 31, 33, 51, and 52 (> 5% of the cases). | Fifty-five (85%) of the 65 hrHPV positive cone specimens contained a single hrHPV infection, 9 (14%) had a double hrHPV infection, and 1 cone contained 4 hrHPV types. Testing for HR HPV identified all recurrent/residual high-grade CIN. Focusing on women with persistent HPV types through genotyping substantially increased positive predictive value but at a loss in sensitivity. |

Abbreviations: HGS-human genetic signatures; BS- additional eight upstream and two downstream primers to GP5+/6+; MPG-multiplexed HPV genotyping; CIN-cervical intraepithelial neoplasia

## Table 1.12: (continued)

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 24 | Castle et al., 2009 | 531 | Linear Array® and cobas 4800 HPV (c4800) | 162/531 (30.5%) both positive; 209/531 (39.4%) both negative; 142/531 (24.9%) HR HPV detected | A very good agreement between the well-validated Linear Array HPV genotyping assay and the new prototype clinical assay, the c4800 test, which offers partial HPV genotyping for HPV16 and HPV18. The reproducibility and reliability of the c4800 test should be established before it can be widely used for cervical cancer screening |
| 25 | Dalstein et al., 2009 | 135 cervical samples positive with HC2 | PCR, Hybrid Capture 2; Linear Array® HPV genotyping vs PapilloCheck test | 91/135 (67.4%) +ve concordant 34/135 (25.1%) –ve concordant 10/135 (7.4%) discordant 58/110 (52.7%) multiple infections | The PapilloCheck test and the Linear Array test give comparable results for detecting HPV in cervical specimens. However, these results also suggest that there is a need to standardize the type-specific sensitivity of genotyping methods and to evaluate their accuracy to detect multiple HPV infections. |
| 26 | Erali et al., 2009 | 197 | Real-time PCR DNA sequencing HPV-QUAD vs Hybrid Capture 2 | 91% concordant (180/197) 9% discordant (17/197) Single infection: 48.6% (53/109) Multiple infections:16.5% (18/109) | The automation conferred with the INFINITI automated system provides a reliable and convenient platform for walkaway analysis in the clinical laboratory. The HPV-QUAD assay chemistry is robust and provides reproducible HPV genotyping results. |
| 27 | Galan-Sanchez and Rodriguez-Iglesias, 2009 | 83 | Hybrid Capture INNO-LiPA vs Linear Array vs Clinical Array | 39.7% concordant (31 samples) 56.4% compatible (44 samples) 3.8% discordant (3 samples) Single infection: 77.4% (24/31) Multiple infections: 22.6% (7/31) | All genotyping methods tested are highly comparable and suitable for clinical and epidemiological studies. The major challenges to HPV typing are to find international standards to evaluate the typing techniques and to make available these technical improvements to developing countries, where the necessity for such tests is a prime concern. |
| 28 | García-Sierra et al., 2009 | 408 | Clinical Array vs Hybrid Capture II Papanicolau smear | 90% concordant (367/408) 246/408 (60.2%) HPV positive 206/246 (83.7%) HR HPV Multiple infections: 50% (123/246) | Screening with Papanicolau smear and HC-II tests, followed by HPV detection and genotyping, provided an optimal identification of women at risk for the development of cervical cancer. The array method also made allowed us to determine the possible contribution of the available vaccines. |
| 29 | Hong et al., 2009 | 258 | Hybrid Capture 2 CM-LA vs DM-LA | 83.1% concordant (207/249) 147/249 (59%) HPV positive | Digene media to be interchangeable with Cytyc media when performing the Roche linear array genotyping test. Clinically, we could perform the Roche linear array genotyping test with the same Digene media among women, positive for HC2 assay. |
| 30 | Iftner et al., 2009 | 11000 | Hybrid Capture II Real-time Multi-plex PCR INNO-LiPA v2 | Overall concordant: INNO-LiPA v2/multiplex PCR, with SurePath, normal (85.7%), ASCUS /LSIL-1 (87.5%), ASCUS/LSIL-2 (92.2%), and HSIL (94.9%) | Analyses of DNA genotype testing compared to cytology results demonstrated a significant discordance between cytology-negative (normal) and HPV DNA-positive results. This demonstrates the challenges of cytological diagnosis and the possibility that a significant number of HPV-infected cells may appear cytologically normal. |

Abbreviations: CM-LA - Cytyc media-based linear array HPV test; DM-LA - Digene media-based linear array HPV test

## Table 1.12: (continued)

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 31 | Jamison et al., 2009 | 175 | PCR<br>Roche Linear Array | 82.9% (145/175) HPV-positive<br>17.1% (30/175) HPV-negative<br>37% (10/27) HPV-positive in normal cytological samples | The assay was able to detect multiple HPV infection with a wide range of genotypes in LBC samples sent for routine cytological analysis. It would be suitable for use in a cytopathology laboratory. |
| 32 | Lee et al., 2009 | 3222 | Nested PCR<br>DNA sequencing | 11% (352/3222) HPV-positive<br>92% (324/352) single infection<br>8% (28/352) multiple infections | Direct automated DNA sequencing is a reliable means for validation of HPV genotyping in a routine clinical microbiology laboratory. However, PCR amplification, the identification of the signature sequence for different genotypes by DNA sequence alignment for genotyping are some of the challenging issues that the laboratory staff must become familiar with. |
| 33 | Meftah el khair et al., 2009 | 89 | PCR<br>Southern Blot | 92% (82/89) HPV-positive<br>56.1% (46/82) single infection<br>44.9% (36/82) multiple infections | HPV-16 and persistent infections with other high-risk HPV types are more likely to progress toward cervical neoplasia, but the present study suggests that infections with multiple types might contribute additional prognostic value. |
| 34 | Schenk et al., 2009 | 123 (101 gyneco-logical, 8 genital warts,7 otorhino-laryngeal lesions, 5 skin warts, 2 orolabial) | Biochip vs<br>CE marked PCR RH (GenID-PCR) | 87.8% concordant (101/115)<br>24.3% (30/123) HR HPV<br>16.2% (20/123) LR HPV | The Biochip showed better performance in the detection of multiple infections of high-risk genotype. Due to the different probe configurations used in the two assays, GenID-PCR achieves only group-specific detection of many HPV genotypes, whereas Biochip allows for specific identification. Overall, the newly developed HPV chip system (Biochip) proved to be a suitable tool for HPV detection and genotyping; it also proved to be superior for establishing HPV genotyping methods. |
| 35 | Seme et al., 2009 | 70 | DG RH Test RUO vs<br>IG E CE | 60% concordant (42/70)<br>40% compatible (28/70)<br>88% HR HPV detected (37/42)<br>7% LR HPV detected (3/42)<br>5% no HPV detected (2/42) | A novel Digene test is suitable for the detection of hr-HPV genotypes in clinical samples and it provides comparable results to the well established INNO-LiPA assay. Although INNO-LiPA identified significantly more samples with multiple HPV genotypes than the Digene test, the clinical benefit of such a difference is at present unclear. |

Abbreviations: LBC-liquid-based cytology; DG – Digene HPV Genotyping;; IG E CE-INNO-LiPA HPV Genotyping Extra CE assay; RH-reverse hybridisation;

**Table 1.13: Comparison among various methods for HPV detection and genotyping in the head and neck**

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 1 | Schwartz et al., 2001 | 254 tissues from OSCC patients | PCR and sequencial hybridisation | 15.1% mucosal HPV 16 detected | The presence of HPV type 16 DNA is independently associated with a favorable prognosis in patients with oral squamous cell carcinoma.<br>HPV genotyping may provide important prognostic information. |
| 2 | Klaassen et al., 2004 | 30 FFPETs<br>100 liquid-based cytology samples | PCR<br>High-density microarray<br>DNA sequencing | 53/130 (41%) mucosal HPVs | A total of 45 HPV types were identified by a single type-specific probe, whereas the remaining 8 mucosal HPV types could be identified by a specific combination of probes. The simple assay format allows usage of this assay without expensive equipment, making it accessible to all diagnostic laboratories with PCR facilities. |
| 3 | Gonzalez et al., 2007 | 77 oral lesions (cases)<br>60 normal oral mucosa (control) | PCR-RFLP-dot blot | 91.0% of HPV- benign lesions<br>14.3% of non-HPV-benign lesions<br>51.5% of preneoplasias<br>60.0% of cancers. | No control sample tested HPV positive. In benign HPV- associated lesions, 30.0% of HPV positive samples harbored high-risk types, while in preneoplastic lesions the value rose to 59.9%. In cancer lesions, HPV detection in verrucous carcinoma was 88.9% and in squamous cell carcinoma 43.8%, with high-risk type rates of 75.5% and 85.6%, respectively. |
| 4 | Mitra et al., 2007 | 92 head & neck and 28 leukoplakia lesions | PCR<br>Southern hybridisation<br>DNA sequencing | HPV positivity was 69%. (59)<br>27 were HPV negative (31%)<br>21% to harbour p53 mutations in the coding region of the gene. | HPV status was identified in 86 samples. HPV status for six samples was not determined because of the unavailability of the DNA samples. It was proposed that genetic and epigenetic alteration of p53 and HPV mediated p53 inactivation possibly follow distinct pathways during the development of HNSCC from normal epithelium via dysplasia. |
| 5 | Schlecht et al., 2007 | 42 fresh frozen tumour samples | PCR and RT-PCR<br>cDNA microarray | HPV 16 was detected in 29% (12/42) of HNSCC tumours by both type specific PCR and RT-PCR. HPV prevalence was highest in pharyngeal tumours (45%) | A total of 13 tumours were positive for HPV16-specific L1, or for the HPV16-E6 gene and URR. Most were positive for HPV16 alone (11/13). Strong RNA expression for HPV16-E6 was observed in all but four HPV16-positive specimens, and three demonstrated moderate levels for the E6*splice variant. An additional three tumours exhibited strong HPV16-E6 activity despite being DNA-negative, and six displayed moderate expression for the E6*splice variant. |
| 6 | Acay et al., 2008 | paraffin-embedded specimens<br>50 oral lesions<br>50 (controls) | CSA-ISH | 24% HPV infection markedly higher than controls. | The overall prevalence in these premalignant and malignant lesions was markedly higher than in the control group, and that high-risk types were the most frequently found within HPV-positive cases, strongly suggests that HPV is likely to play a role in oral carcinogenesis. |
| 7 | Fujita et al., 2008 | paraffin-embedded specimens<br>23 verrucous carcinomas (VCs)<br>10 (controls) | ISH-PCR-IHC | 11 (48%) had HPV-DNA (by PCR)<br>6 (26%) had intranuclear HPV in the upper portion of the squamous epithelium (by ISH). | Nine of the 11 PCR-positive specimens showed multiple infections with low- and high-risk HPVs. No HPV could be found in control specimens by ISH.<br>Multiple infections with low- and high-risk HPVs and their rapid replication during hyperkeratinization may participate in the histogenesis of oral VC. Oral VC tumorigenesis may involve the inactivation of p53, which is associated with HPV infection. |

Abbreviations: FFPETs - formalin-fixed paraffin-embedded tissues; URR – upstream regulatory region; CSA-ISH – *In situ* hybridisation with signal amplification; IHC – immunohistochemistry; RFLP – random fragment length polymorphism

**Table 1.13: (continued)**

| No. | Reference (First author, year) | N (actual sample size) | Method | Specificity/Sensitivity (especially high-risk HPVs) | Findings/Conclusions |
|---|---|---|---|---|---|
| 8 | Lohavanichbutr et al., 2009 | 119 patients with primary OSCC 35 patients without cancer (controls) | PCR Roche LINEAR ARRAY HPV Genotyping Test | 41/119 tumours (34.5%) HPV positive. 2/35 normal tissue samples (5.7%) HPV positive; 39/43 (91%) HPV specimens were HPV type 16. | A higher prevalence of HPV DNA was found in oropharyngeal cancer (23 of 31) than in oral cavity cancer (18 of 88). The differences in the biology of HPV-positive and HPV-negative oropharyngeal cancer may have implications for the management of patients with these different tumors. |

Abbreviations: FFPETs - formalin-fixed paraffin-embedded tissues; URR – upstream regulatory region; ISH – in situ hybridisation; IHC – immunohistochemistry; RFLP – random fragment length polymorphism

### 1.2.6 Meta-analysis of HPV involvement in specified cancer types

#### 1.2.6.1 HPV in head and neck cancer

A compilation of 94 case reports and a clinical series over the past 18 years were included in this comprehensive meta-analysis involving diverse HPV detection systems and types of samples used and comparing their efficiency in discriminating amongst normal oral mucosa, potentially malignant lesions and carcinomas. They made the very conservative generalisation that regardless of the chosen HPV detection systems, and sample types analysed, the likelihood of detecting HPV was 2-3 times higher in precancerous oral mucosa and 4.7 times more in oral carcinoma relatively compared to normal oral mucosa (Miller and Johnstone, 2001). A meta-analysis was carried out targeting any association that may exist between oral carcinogenesis and HPV infection in a Chinese ethnic group following inclusive criteria, case-control studies (from 1990 to 2003) and PCR techniques finalised 10 studies at the end. Overall HPVs (regardless HPV types) were more likely to be detected at 8.89 times higher in OSCC compared in normal oral mucosa. On the other hand, HPV16 was positively identified at 6.81 times higher in OSCC compared in normal oral mucosa specimens (Wang et al., 2004).

Hobbs et al. (2006) systematically selected seventeen studies (unmatched case-control and matched analysis) to compare HPV16 exposure among site-specific head and neck cancer. They successfully outlined the magnitude of the association between HPV16 infection and anatomical sites in descending order as follows: tonsil (the strongest), oropharynx (intermediate), and the weakest for oral and larynx. In meta-analysis involving 37 studies, patients with HPV-positive HNSCC was associated with

an improved overall survival (OS) (Hazard ratio, HR: 0.85) and disease-free survival (DFS) (HR: 0.62) than HPV-negative HNSCC patients. In oropharyngeal cases, patients with HPV-positive was associated with an improved OS (HR: 0.72) than HPV-negative patients. In non-oropharyngeal cases, patients with HPV-positive and HPV-negative seem to be comparable in OS (Ragin and Taioli, 2007).

### 1.2.7 Reviews of head and neck cancer and the role of HPV in oral cancer

Syrjänen (2003) suggested that the two most prominent candidates of HPV types in potentially malignant and malignant lesions were HPV 16 and HPV 18. Whilst HPV 6 and HPV 11 could be connected with benign papillomatous lesions of the oral mucosa (Syrjänen, 2003). It was noted that strong and convincing experimental data are required to support the proposed "hit and run" theory and to unravel HPV mechanism of infection in oral carcinoma (Syrjänen, 2003). Despite the establishment of HPVs as an independent risk factor for oral cancer, its involvement in laryngeal cases is still unresolved and controversy still exists (Syrjänen, 2005).

Nowadays, HNSCC are being treated via combined treatment modalities but still there are confounding issues i.e. suitable dose, reagent sequences and to precisely set the usage of biologically active agents (Mao et al., 2004). More than 90% head and neck cancers are SCCs and alcohol and smoking are the major risk factors and have an additive effect. Multi-modality treatment with surgery or radiotherapy is limited to early-stage tumours and concurrent chemoradiation to locally advanced unresectable tumours (Marur and Forastiere, 2008). Generalisation could be made about a new trend of HPV-positive prevalence towards younger individuals, male patients who either do

not smoke or smoke less and have a low alcohol intake (Fakhry and Gillison, 2006; Adelstein and Rodriguez, 2010; Lajer and Buchwald, 2010).

### 1.2.8 Novel studies or reviews on HPVs involvement in various sites

#### 1.2.8.1 HPVs in cutaneous squamous cell carcinomas

Purdie et al. (1993) established the presence of episomal HPV in cultured keratinocytes from cutaneous lesions of renal allograft patients. The nature of viral episomic loss observed from this study could be overcome by maintaining serial passages of cultures until senescence (William et al., 1994). Utilising the newly developed combination of degenerate primer PCR, we are able to identify multiple HPV infections from mucosal and cutaneous biopsies (Surentheran et al., 1998). Harwood et al. (1999) successfully improved HPV detection in wart samples using degenerate and nested PCR approaches and the incidence ranges from 27-84%. Another study provided further support for the role of Epidermodysplasia verruciformis (EV)-type HPV in cutaneous lesions (Purdie et al., 2005). Despite facing a great challenge in identifying multiple HPV infections that exist in a single sample (either from eyebrow hairs or paraffin-embedded skin biopsies), a newly applied method by combining broad-spectrum PCR (PM-PCR) and reverse hybridisation assay (RHA) promises the detection of 25-beta HPV types in a single reaction (de Koning et al., 2006). A 6-month period of beta papillomaviruses persistence in the skin of healthy individuals might be set as baseline guidance to understand the mechanism of malignant transformation to be elucidated by future studies (de Koning et al., 2007).

Casabonne et al. (2007) in their prospective case-control study involving 39 cutaneous SCC cases (selected from the actual population size of 65,429 recruited people and another fresh blood samples provided by 19,500 candidates) were unable to demonstrate a strong link with HPV presence but did not exclude the possibility of HPV-UV interaction causing cell immortalisation, as suggested from a previous study. The low prevalence of cutaneous HPV types observed in another case-control study still unravelled cutaneous HPV’s role in the aetiology of ocular surface squamous neoplasia (de Koning et al., 2008). In their review, Feltkamp and co-workers (2008) suggested that epidemiological studies involving UV exposure at multiple latitudes might provide a true indication of its association with HPV prevalence, which were mainly beta types, in carcinogenesis of the skin (Feltkamp et al., 2008).

#### 1.2.8.2 HPVs in cervical squamous cell carcinomas

The novel Short PCR Fragments (SPF) system was claimed amongst the most efficient approaches in identifying, at minimum, simultaneously 43 different HPV genotypes in a single reaction in cervical specimens (Kleter et al., 1998). There was no doubt that by combining previously reported methods with RHA, improved the detection rate and was less laborious and might be an option for routine HPV screening of cervical scrapes (Melchers et al., 1999; Kleter et al., 1999; Fey and Beal, 2004; Khan et al., 2005). In addition, this approach appeared more sensitive relatively than PCR-DNA sequencing techniques in handling cases related to multiple infections of HPV in a single sample (Kleter et al., 1999; Van Doorn et al., 2006; Castle et al., 2008). By exploiting $T_m$ differential in real-time PCR, the distinction between multiple high-risk HPV infections could be made, specifically between HPV 16 and HPV 18 infections and/or with other

non-oncogenic HPV types and this is truly beneficial in mass screening of cervical specimens for HPV (Cubie et al., 2001).

Wentzensen and colleagues in their systematic review revealed that random integration of HPV occurred within the host genome but its effect on the functionality of crucial cellular genes was not observed in anogenital tract specimens (Wentzensen et al., 2004). There are advantages with repeated HPV genotyping especially for type specific identification of persistent HPV infections, but related cost-benefit analysis on a larger scale are required before being taken into routine practice (Cuschieri et al., 2005).

#### 1.2.8.3 HPV in oesophageal squamous cell carcinomas

Si et al. (2005) demonstrated that the integration of high-risk HPV, particularly HPV 16, into the host genome was frequently present in oesophageal squamous carcinoma (ESCC) as it was previously reported in cervical cancer. The existence of cutaneous EV-HPV type 5 together with mucosal HPV 16 in oesophageal cancer has been reported but the actual role of the former is still disputed (Saravanan et al., 2006).

#### 1.2.8.4 Cancer of the oropharynx

HPV DNA was consistently detected in a substantial fraction of cancers of the oropharynx with an estimated average prevalence of 35%. HPV16 DNA was detected in approximately 80% of HPV-positive cases. Several studies that compared tumours with normal tissue revealed large differences in HPV DNA detection, particularly that of

HPV16 in cancer of the tonsil. Seroepidemiological case–control studies and one prospective study showed marked increases in risk associated with serological markers of expression of HPV. These associations were much stronger than those observed for cancer of the oral cavity.

#### 1.2.8.5 Cancer of the larynx

HPV DNA was detected in a variable fraction of cancers of the larynx. Limited and contradictory data resulted from comparisons of tumours and normal tissue. Some cross-sectional and prospective seroepidemiological data suggested a modest association with HPV16 and 18. In patients with recurrent papillomatosis, some well documented reports pointed to an involvement of HPV6 and 11.

## 1.3 p16$^{INK4a}$, A TUMOUR SUPPRESSOR GENE

### 1.3.1 Genetic background

The G1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first 'gap' phase (G1) into the DNA synthesis phase (S). Two cell cycle cyclin-dependent kinases, CDK 4/CDK 6, cyclin D and the transcription complex that includes retinoblastoma protein (Rb) and E2F are pivotal in controlling this checkpoint (Rocco and Sidransky, 2001). During G1 phase, hypophosporylated RB (pRB) binds to the E2F transcription factors, inhibiting the downstream transcription (Doeberitz, 2002). The complexes formed between the former to cyclin-D, CDK 4 and/or CDK 6 trigger phosphorylation of pRB. At this stage, pRB releases E2F, permitting transcription of S-phase genes encoding for proteins that amplify the G1 to S phase switch and that are required for DNA replication (Schafer, 1998; Nagpal and Das, 2003) (Figure 1.4).

The Human Genome Organisation nomenclature committee assigned the designation *CDKN2A* (for *c*yclin *d*ependent *k*inase i*n*hibitor 2A) for p16$^{INK4a}$. This gene is located on chromosome 9p21 and lies on three exons (Foulkes et al., 1997). *CDKN2A* encodes a 156 amino acid with $M_r$ 16, 569 cell cycle inhibitor protein (Ruas and Peter, 1998). In normal cells, it binds to the non-catalytic side of CDK 4 and/or CDK 6 (Figure 1.4). The tumour suppressive action of p16 inhibits the catalytic activity of CDK4-6/cyclin D1 enzyme complex, required for hypophosphorylated retinoblastoma protein (pRB) and cell cycle progression. Thus, it blocks abnormal cellular division and proliferation (Serrano et al., 1993). Ultimately, the cell cycle at the G1 phase is halted due to the enzyme activity that has been inhibited (Giordano et al., 2008).

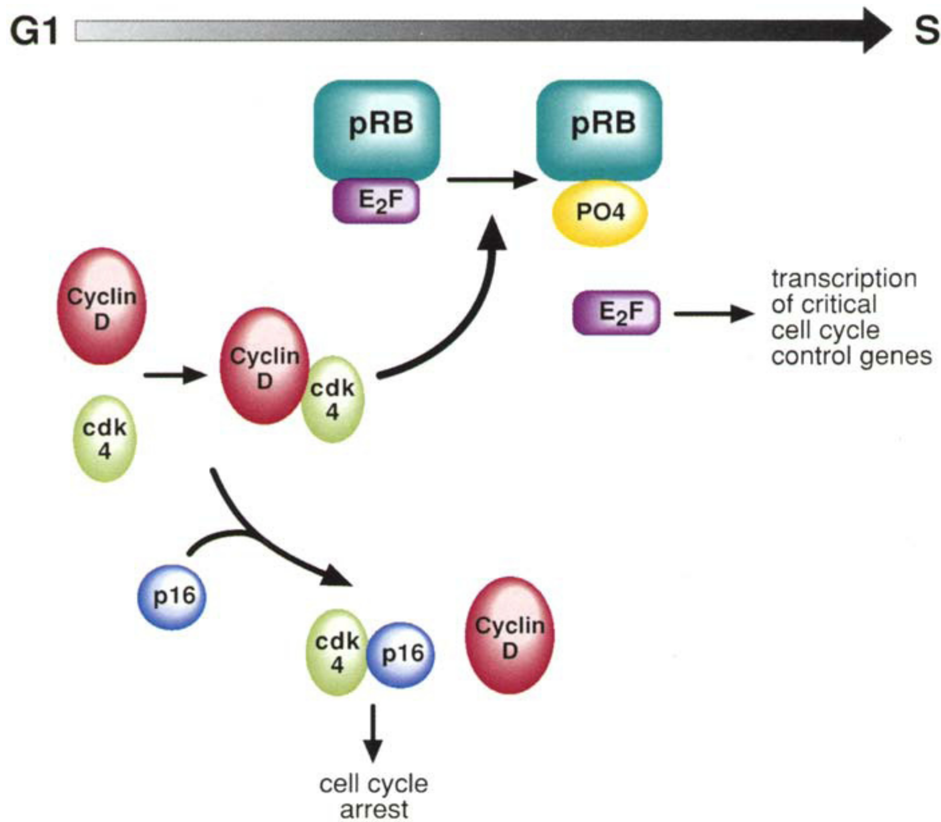



**Figure 1.4: p16 roles in cell cycle regulation**
Reprinted with permission from Liggett and Sidransky (Liggett and Sidransky, 1998).

Free cyclin D1 is degraded by a ubiquitin-dependent proteosome pathway if there are increased levels of p16. In addition, p16 also has a dual role by inhibiting E2F-DB active transcriptional complex formation and at the same time promoting Rb-E2F repressive transcriptional complex formation. The consequence is that p16 restricts the progress of cell division at the G1/S checkpoint by preventing E2F-dependent transcription (Rocco and Sidransky, 2001). Many different stimuli exert checkpoint control including TGFb, DNA damage, contact inhibition, replicative senescence, and growth factor withdrawal. The first four act by inducing members of the INK4 or Kip/Cip families of cell cycle kinase inhibitors (Schwart and Shah, 2005).

### 1.3.2 p16 and oral cancer

Results from an *in vitro* study suggested that early genetic alterations involving the functional loss of tumour suppressor genes i.e. p16 and p53 (lies on locus 9p21 and 17p13, respectively) were associated with oral cancer progression (Prime et al., 1997). In addition, Partridge and colleagues found deletions located centromeric and telomeric to the p16 gene at 9p21 in OSCC (Partridge et al., 1999). In contrast with another study, no p16 genetic alterations were observed in HPV-positive cases associated with betel chewing practices (Heinzel et al., 1996) or tobacco smoking exposure of oral cancer patients (Lazarus et al., 1998). Nevertheless, inactivation of p16 expression due to alteration of the α transcript was commonly observed in OSCC and was consistent with previous findings (Akanuma et al., 1999).

The postulated sequence of malignant transformation events in oral carcinogenesis is based on the occurrence of aberrations in descending order, 9p21 (encodes for p16) and 3p (encodes for several tumour suppressor genes, TSGs) and 17p13 (encodes for p53) (Scully et al., 2000). Despite no association having been found between the p16 gene alterations with cancer stage and cancer site, an association was found between p16 and cancer metastasis to regional neck nodes (Tsai et al., 2001). Therefore, screening for both cyclin D1 and p16 aberrations in OSCCs may be useful for identifying aggressive tumours, disease recurrence and in patients with a poor prognosis (Uzawa et al., 2007). The expression of $p16^{INK4a}$ was associated with OSCCs (Suzuki et. al., 2006; Muirhead et al., 2006). Similarly, Greer Jr. et al., (2008) found that $p16^{INK4a}$ expression was strong in all 45 OSCC samples tested. Commonly, p16 is inactivated by deletions, mutations or promoter methylation in the absence of HPV in

HNSCC (Shintani et al., 2001). Table 1.14 shows the proportion of p16 expression among selected studies.

**Table 1.14: The percentage of p16 expression among studies**

| No. | Investigator | *N* | Type of sample | p16 expression (%) | Remark |
|---|---|---|---|---|---|
| 1. | Sathyan et al., 2006 | 241 | 147 buccal carcinoma<br>94 tongue carcinoma | 28% of buccal<br>47% of tongue | p16 expression could support conventional prognostic markers in buccal mucosa |
| 2. | Muirhead et al., 2006 | 45 | OSCC | 13% | p16 expression was associated with the status of keratinisation and differentiation |
| 3. | Suzuki et al., 2006 | 66 | OSCC | 21% without lymph node metastasis<br>6% with metastasis | $p16^{INK4a}$ is a suitable marker for OSCC |
| 4. | Greer Jr. et al., 2008 | 140 | 81 STK<br>29 SCC<br>30 benign alveolar ridge keratoses (ARK) | 35% grade I STK<br>44% grade II STK<br>43% grade III STK<br>52% SCC<br>20% ARK<br>0% normal | p16 is suitable for assessing SCC and dysplasia but not for STK lesions |
| 5. | Buajeeb et al., 2009 | 56 | 16 OSCC,<br>15 OL (with/without dysplasia) and normal | 18.75% OSCC<br>26.7% OL | p16 is not reliable as a marker |

*N* – number of samples; STK-smokeless tobacco keratosis

### 1.3.3 p16 promoter hypermethylation

Alteration of p16 mRNA expression as a result of methylation of the p16 gene promoter region was observed in oral rinses from leukoplakia patients (Lopez et al., 2003) and in primary OSCCs (Akanuma et al., 1999; Shintani et al., 2001; Nakahara et al., 2001; Yakushiji et al., 2003; Lee et al., 2004 and Ohta et al., 2009). However, Nakashini et al, (1997), did not observe p16 hypermethylation in OSCCs. Inactivation of the p16 gene by CpG methylation was most unlikely to occur in normal human oral keratinocytes (Cody et al., 1999). Nevertheless, CpG methylation was associated with malignant

transformation in mild or moderate epithelial dysplasia (Cao et al., 2009). Hypermethylation of p16 due to pathogenesis of potentially malignant oral lesions was associated with betel-quid (Lin et al., 2000; Tran et al., 2005; Takeshima et al., 2008; Yoshihiro et al., 2008), chewing tobacco (Kulkarni and Saranath, 2004) and tobacco smoking (Hasegawa et al., 2002; Saatci et al., 2009).

Ishida and colleagues found that concurrent promoter hypermethylation of p16 and p14 were significant and directly associated with clinical parameters of OSCC (Ishida et al., 2005). In contrast, p16 and p14 both exhibited opposing clinical effects of gene methylation in oral carcinoma (Sailasree et al., 2008). In addition, p16 methylation was found to be independent from tumour stage and specific location in the oral cavity (Viswanathan et al., 2003). Hasegawa et al. 2002, on the other hand, noted that p16 hypermethylation was directly associated with tumour size but that did not exhibit lymph node metastasis. Guo 2007, revealed that promoter hypermethylation results in inactivation of p16 tumour suppressor gene in mucoepidermoid carcinoma (MEC), human salivary glands (Guo et al., 2007). A similar scenario was observed in saliva DNA where abnormal p16 promoter methylation occurred and at a higher percentage especially near to the oral cavity (Rosas et al., 2001). Kresty et al. (2002) found that the highest p16 hypermethylation presented at the tongue and the floor of the mouth of patients with severe epithelial dysplasia.

CpG site methylation was found in the p16 gene promoter in carcinoma specimens and this was highly tumour specific (Shaw et al., 2006; Shaw et al., 2007). It was believed that p16 promoter hypermethylation would cause its inactivation and that it occurred at the early stage of OSCC carcinogenesis (Von Ziedler et al., 2004; Kato et

al., 2006; Ruesga et al., 2007 and Šupić et al., 2009). Table 1.15 shows the proportion of p16 promoter hypermethylation observed among 30 studies and the specific method used for assessment. It was noted that the occurance of this event in OSCC cases varied among studies from 0-90% depending on the method of detection. No p16 hypermethylation was observed in normal oral specimens (controls) in appropriate studies.

The loss of p16 expression as a result of promoter hypermethylation is an early event in oral carcinoma and a useful biomarker for predicting local recurrence in carcinoma of the tongue (Sinha et al., 2009). Nakahara et al., (2006) reported that p16 hypermethylation could also be observed in the serum of recurrent OSCC by using a methylation specific-PCR (MS-PCR) technique. However, the role of p16 hypermethylation as a predictive risk factor for OSCC or disease recurrence remains unclear and contradictory (Ruesga et al., 2007). In addition, p16 methylation did not associate with HPV-induced tumours (pathogenic mechanism) and affect protein expression in cervical carcinoma but was more related to genetics and epigenetic instability (Nehls et al., 2008).

**Table 1.15: The prevalence of p16 promoter hypermethylation among studies**

| No. | Investigator | Region | Method | *N* | % of PH[1] |
|---|---|---|---|---|---|
| 1 | Nakashini et al., 1997 | Japan | n/a | 50 | 0% |
| 2 | Akanuma et al., 1999 | Japan | MS-PCR[2] | 14 | 28.6% |
| 3 | Lin et al., 2000 | Taiwan | RFMA[3] | 110 | 54% *tongue*<br>22% *other sites* |
| 4 | Nakahara et al., 2001 | Japan | MS-PCR | 32 | 50% |
| 5 | Shintani et al., 2001 | Japan | MS-PCR | 32 | 50% |
| 6 | Rosas et al., 2001 | Brazil | MS-PCR | 30 | 47% |
| 7 | Kresty et al., 2002 | USA | MS-PCR | 28 | 57.7%*<br>*severe oral dysplasia* |
| 8 | Hasegawa et al., 2002 | USA | MS-PCR | 80 | 32.5% *HNSCC* |
| 9 | Viswanathan et al., 2003 | India | MS-PCR | 99 | 23% |
| 10 | Lopez et al., 2003 | Spain | MS-PCR | 34 | 44%<br>*Leukoplakia* |
| 11 | Yakushiji et al., 2003 | Japan | n/a | 25 | 48% |
| 12 | Kulkarni and Saranath, 2004 | India | MS-PCR | 60 | 66.7% |
| 13 | Von Zeidler et al., 2004 | Brazil | MS-PCR | 258 | 9.7%<br>*normal posterior tongue border* |
| 14 | Ishida et al., 2005 | Japan | MS-PCR | 49 | 34.7% |
| 15 | Tran et al., 2005 | Japan | MS-PCR | 27 | 63% |
| 16 | Kato et al., 2006 | Japan | MS-PCR | 55 | 50.9%<br>*mixed OSCC, VC and Cis* |
| 17 | Nakahara et al., 2006 | Japan | MS-PCR | 17 | 64.7% |
| 18 | Shaw et al., 2006 | UK | Pyrosequencing | 79 | 28% |
| 19 | Guo et al., 2007 | China | MS-PCR | 38 | 34.2%<br>*MEC salivary glands* |
| 20 | Ruesga et al., 2007 | Spain | MS-PCR | 145 | 20% |
| 21 | Shaw et al., 2007 | UK | Pyrosequencing | 20 | 90% |
| 22 | Guerrero et al., 2008 | Spain | MS-PCR | 24 | 38%<br>*penile SCC* |
| 22 | Nehls et al., 2008 | Germany | MS-PCR | 70 | 25.7%<br>*cervical carcinomas* |
| 23 | Sailasree et al., 2008 | India | MS-PCR | 116 | 29% |
| 24 | Takeshima et al., 2008 | Sri Lanka | MS-PCR | 64 | 73%<br>*mild/severe dysplasia* |
| 25 | Hall et al., 2008 | UK | Pyrosequencing | 284 | 1-26%<br>*epithelial dysplasia* |
| 26 | Šupić et al., 2009 | Serbia | MS-PCR | 77 | 58.4% |
| 27 | Cao et al., 2009 | China | MS-PCR | 78 | 41%<br>*epithelial dysplasia* |
| 28 | Ohta et al., 2009 | USA | MS-PCR | 44 | 63.6% |
| 29 | Saacti et al., 2009 | Turkey | MS-PCR | 42 | 31.8% *MP users*<br>25% *tobacco users* |
| 30 | Sinha et al., 2009 | India | MS-PCR | 38 | 86.8% |

[1]% of promoter hypermethylation (PH) in OSCC unless stated; [2]MS-PCR – Methylation specific-PCR; [3] RFMP – Restriction Fragment Methylation Analysis; VC – verracous carcinoma; Cis – Carcinoma in situ; MEC – Mucoepidermoid carcinoma; MP – maras powder ; *N* – number of samples

### 1.3.4 Was there a link between p16 expression and HPV infection?

Fregonesi et al. (2003) suggested that p16 over-expression occurred as a result of either the failure of this tumour suppressor protein or due to high-risk HPV integration into the host genome. The phenomenon of p16 over-expression found in the nucleus and cytoplasm was highly associated with HPV positivity and was speculated to be unique for HPV positive oropharyngeal carcinoma (Hafkamp et al., 2003; Li et al., 2003). As p16 over-expression is very rarely seen in HPV negative HNSCC and that functional inactivation of Rb by E7 induces p16 upregulation, it is considered a surrogate marker for HPV infection in OPSCC. Figure 1.5 depicts a putative mechanism on the over-expression of p16 in response to the present of HPV particularly HR-HPV.

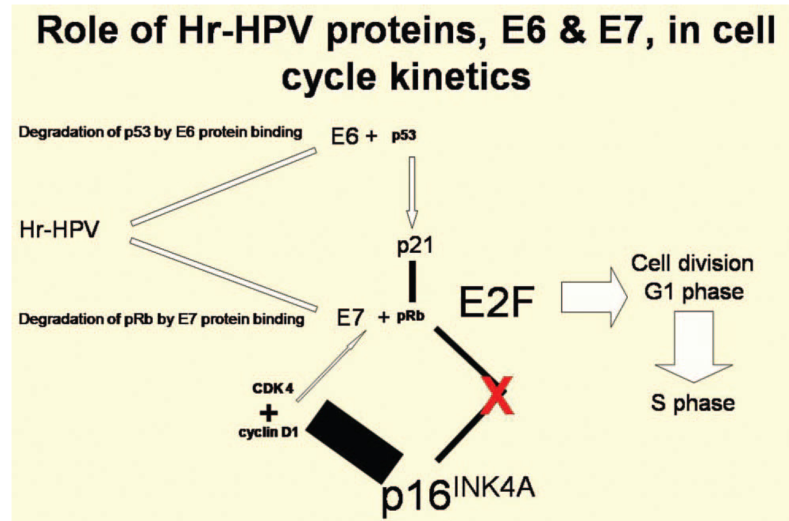



**Figure 1.5: The postulated mechanism on upregulation of p16 in HPV infection.**
(Mulvany et al., 2008)

It was later concluded that p16 was a potential biomarker to indicate the presence of high-risk HPV infection in oral cancer. Other studies clearly observed over-expression of p16$^{INK4a}$ on HPV-induced high-grade oral squamous dysplasia or carcinoma (Cunningham et al., 2006), penile sarcomatoid carcinoma (Poblet et al.,

2008), uterine cervical lesions (Yoshida et al., 2008) and cervical carcinoma (Fujii et al., 2008; Ozgul et al., 2008). A study that was carried out on tonsillar squamous cell carcinomas strongly supported the relationship between HPV16 integration with p16 over-expression (Charfi et al., 2008; Hafkamp et al., 2008; Kuo et al., 2008; Klussmann et al., 2003, 2009).

It was demonstrated that $p16^{INK4a}$ was a sensitive predictor in vulval carcinomas positive for mucosal HPV (de Koning et al., 2008) and especially high risk-HPV (Hoevenaars et al., 2008). p16 over-expression has a significant correlation with HR HPV especially involving HPV16 infections in oropharyngeal cancer (Preuss et al., 2008; Nichols et al., 2009). Moreover, $p16^{INK4a}$ was an indirect marker candidate for cell cycle dysregulation due to its association with high risk-HPV infections in cervical dysplasia and carcinomas (Mulvany et al., 2008). Nevertheless, in laryngeal cases, it was suggested that other factors may take precedence (Laco et al., 2008) because the association was not as strong as in cervical neoplastic lesions (Duncan et al., 2008). A recent study to assess HPV presence found that the combination of a strong HPV signal by pyrosequencing approach and strong $p16^{INKa}$ positive staining gave the best interpretation for relapse and survival in HNSCC (Kong et al., 2009).

However, contradictory findings have shown that over-expression of $p16^{INK4a}$ proteins in OSCC did not correlate with HR-HPV types (Nemes et al., 2006). In addition, very high p16 expression observed not only in HPV positive groups but also in other groups in the absence of HPVs. Therefore, it revealed unconvincing support for previous claims on the HPV-p16 relationship (Smeets et al., 2007; Aulmann et al., 2008; Bohn et al., 2008 and Samama et al., 2008). Another study demonstrated that p16

expression might not be a good predictor of HPV status but a fairly reliable determinant for epithelial atypia severity (Braganca et al., 2008). Carcinogenesis of HPV did not show convincing association with over-expression of p16 in oral smokeless tobacco keratosis (STK), non-neoplastic lesions (Greer Jr. et al., 2008) and female genital tract neoplasm (Giordano et al., 2008). Smith et al. (2008) were unable to get conclusive evidence of this association for survival and disease recurrence for head and neck cancer due to the combined HPV/p16 biomarkers data differ if both were assessed separately. Nowadays, the role of p16 in human cancer is more complex and it related to loss-of-function mutations as cancer progression to advance stage (Rocco and Sidransky, 2001).

### 1.3.5 Is p16 a surrogate marker for high risk-HPV in oral cancer?

Despite the current advancement in molecular technology, the future diagnostic role of p16 was still hampered by a few vital elements that urgently needed to be resolved. As many published articles leave behind very constructive take home messages that could be generalised and quoted as follows: Interpretation variability observed in staining results, sampling convenience and defective gold standard amongst studies. The above question should be answered with caution in order to justify with various sources of evidence to support very convincing and conclusive findings (Table 1.16 and 1.17). Throughout the proposed study, we will try to unravel this mystery by exploiting our recent knowledge and persistent belief in this area and to explore its maximum potential for the benefit of human oral cancer prognosis.

**Table 1.16: An association observed between p16 (IHC) and HPV (HR) expression among studies**

| No | Investigator | Sample origin | HPV/p16 detection | HPV types |
|---|---|---|---|---|
| 1 | Singhi and Westra, 2010 | Head and neck | ISH/IHC | HPV16 |
| 2 | Al-Swiahb et al., 2010 | Oropharyngeal | PCR/IHC | HPV |
| 3 | Smith et al., 2010 | Head and neck | PCR/IHC | HR-HPV |
| 4 | Laksmi et al., 2009 | Cervical | ISH/IHC | HR-HPV |
| 5 | Kumar et al., 2008 | Oropharyngeal | PCR/IHC | HPV16 |
| 6 | Smith et al., 2008 | Head and neck | PCR/IHC | HR-HPV |
| 7 | Hafkamp et al., 2008 | Tonsillar | FISH/IHC | HPV16 |
| 8 | König et al., 2007 | Head and neck | ISH/IHC | HPV16, HPV18 |
| 9 | Lambert et al., 2006 | Cervical | PCR/IHC | HPV16/18/31/33 |
| 10 | Cunningham et al., 2006 | Oral dysplasia | PCR/IHC | HPV16 |
| 11 | Tsai et al., 2005 | Cervical | ISH/IHC | HPV16, HPV18 |
| 12 | Wittekindt et al., 2005 | Tonsillar | - /IHC | HR-HPV |
| 13 | Begum et al., 2005 | Tonsillar | ISH/IHC | HPV16 |
| 14 | Fregonesi et al., 2003 | OSCC | ISH/IHC | HPV16, HPV18 |
| 15 | Li et al., 2004 | Tonsillar | PCR/IHC | HPV |
| 16 | Lu et al., 2003 | Anorectal | BS-HPV/IHC | HPV16 |
| 17 | Klussmann et al., 2003 | Tonsillar | PCR/IHC | HPV16, HPV18 |
| 18 | Sano et al., 2002 | Cervical/dysplasia | ISH/IHC | HPV |
| 19 | Sano et al., 1998 | Cervical | ISH/IHC | HPV16, HPV18 |

Abbreviation: ISH – in situ hybridisation; IHC – immunohistochemistry; BS-HPV – Broad spectrum-HPV

**Table 1.17: No association observed between p16 (IHC) and HPV (HR) expression among studies**

| No | Investigator | Sample origin | HPV/p16 detection | HPV types |
|---|---|---|---|---|
| 1 | Klingenberg et al., 2010 | Tonsillar | PCR/IHC | HPV16, 18 |
| 2 | Friedrich et al., 2010 | OSCC | PCR/IHC | HPV6, 11, 16 |
| 3 | Cao et al., 2010 | Head and neck | ISH/IHC | HPV16 |
| 4 | Greer Jr. et al., 2008 | Oral dysplasia | PCR/IHC | HPV |
| 5 | Galmiche et al., 2006 | Cervical dysplasia | - /IHC | HR-HPV |
| 6 | Nemes et al., 2006 | OSCC | ISH/IHC | HPV |
| 7 | Samama et al., 2006 | Anogenital | ISH/IHC | HR-HPV |
| 8 | Hashi et al., 2006 | Endocervical | PCR/IHC | HPV |
| 9 | Munirajan et al., 1998 | Uterine cervix | PCR-SSCP/RE | HPV16, HPV18 |

Abbreviation: SSCP – single strand conformation polymorphism; RE – restriction enzyme

### 1.3.6 p16 immunohistochemistry and its semi-quantitative assessment

Numerous studies have made an effort to utilise immunohistochemistry to assess the expression of p16 in diverse types of specimens. Generally, we have to admit at this point that there was no consensus agreement amongst investigators in relation to scoring criteria for p16 positivity either via quantitative or qualitative assessments. Table 1.18 shows the summary of selected studies using semi-quantitative assessment and the parameters.

## Table 1.18: Comparison of p16 IHC semi-quantitative scoring amongst studies

| Reference | Sample types (oral) | IHC Kit | IHC scoring method |
| --- | --- | --- | --- |
| Fischer et al., 2010 | 5-µm section FFPET; 365 blocks of OPSCC punched biopsies | Hematoxylin p16$^{INK4a}$ (ABC-Elite, Vector Laboratories, Burlingame, CA) | Positive:<br>The percentage of nuclear staining of the tumour over total number of tumour cells (nuclei)<br>≥ 5% immunoreactivity in tumour cells.<br>The score between two independent observers were averaged<br>No staining intensity was assessed |
| Buajeeb et al., 2009 | 56 samples including OSCC, OL with or without dysplasia and normal oral mucosa | Hematoxylin p16$^{INK4a}$ (DakoCytomation) 1:25 DakoCytomation Target Retrieval Solution in steamer | Positive:<br>When nuclear and/or cytoplasmic staining was compatible with that of positive control.<br>Five or more stained cells. All positive cells and all cells in the specimens were counted, calculated – percentage of positive cells.<br>The staining intensity:<br>Weak, moderate or strong when compared with the positive control.<br>10 Random areas were counted. At least 500 cells were counted in each case. |
| Greer Jr. et al., 2008 | 140 tissues; 81 STK cases, 29 SCC and 30 cases of benign alveolar ridge keratoses (ARK)<br>*Negative control:*<br>1 mg/ml of subclass matched IgG 1k | DAKO autostainer<br>Indirect avidin-biotin immunoperoxidase<br>Normal horse serum<br>p16$^{INK4a}$ Ab-4 antibody (16PO4) | Positive:<br>Brown nuclear and cytoplasmic staining. The proportion of positively staining and the strength of staining to produce a semi-quantitative scoring standard.<br>0 – staining was similar to background<br>1+ - just slightly darker than the background<br>2+ - clearly darker than the background and there was relatively strong staining of cytoplasmic and nuclei<br>3+ - intense staining sections (dark brown staining)<br>To quantify the proportion of cells with positive result<br>1+ - 5-35%<br>2+ - 36-75%<br>3+ > 75% |
| Angiero et al., 2008 | 5-µm section FFPET; 54 oral cavity biopsies | p16 (clone E6H12, dilution 1:40; Novocastra, Newcastle, UK | Positive:<br>The relative number of nuclei stained and their localisation (upper 2/3 of the epithelium) ; The areas of infiltration for invasive carcinoma<br>≥ 10% of nuclei were stained |
| Muirhead et al., 2006 | 5-µm section FFPETs; 45 oral cavity squamous carcinomas | Hematoxylin p16$^{INK4a}$ (DakoCytomation) 1:25 DakoCytomation Target Retrieval Solution in steamer | p16 was not observed in normal mucosa<br>The amount of staining<br>0 – no staining<br>1 – staining up to 24% of tumour cell of nuclei<br>2 – 25-49% of nuclei<br>3 – 50-74% of nuclei<br>4 – staining of > 75% of nuclei |

**Table 1.18: (continued)**

| Reference | Sample types (non-oral) | IHC Kit | IHC scoring method |
|---|---|---|---|
| Kok et.al., 2010 | 5-µm section FFPETs; 35 hysterectomy specimens; 14 endocervical adenocarcinomas (ECAs) and 21 endometrial adenocarcinomas (EMAs)<br>*Positive control:*<br>Tissues of SCC from uterine cervix<br>*Negative control:*<br>Excluding primary antibody | $p16^{INK4a}$ (F12, sc-1661, Santa Cruz)<br>1:200<br>pre-treatment: microwave with citrate buffer (pH 6.0)<br>Streptavidin peroxidase method | Independent cytoplasmic stain alone, irrespective of the nuclear stain, and vice versa<br>German semi-quantitative scoring system (staining intensity and staining extent):<br>Staining intensity<br>0 – no stain<br>1 – weak stain<br>2 – moderate stain<br>3 – strong stain<br>Staining extent<br>0 – 0%<br>1 – 1-10%<br>2 – 11-50%<br>3 – 51-80%<br>4 – 81-100%<br>Final immunoreactive score – by multiplying the three-tier intensity score with the four-tier extent of positive staining score in the tumour (min 0, max 12). |
| Tsoumpou et al., 2009<br>REVIEW PAPER | 61 studies; 27 cervical cytological specimens (6 studies the biomarkers in histological specimens), 34 staining only in histological samples | | Classification proposed by Klaes and colleagues:<br>Negative - <1% of the cells were positive<br>Sporadic - <5%, isolated cells were positive<br>Focal - <25% of the cells were positive, small clusters<br>Diffuse - >25% of the cells were stained positive<br>Positive cut-off for p16 – the diffuse stain that includes both basal and parabasal cell layers and indicates hr-HPV-induced transformation |
| Bradley et al., 2006 | 4-µm section FFPETs; 119 biopsies<br>*Positive control:*<br>Vaginal squamous cell carcinoma sections<br>*Negative control:*<br>$1^{o}$ antibody replaced by buffer | H & E<br>Heat-induced (electric pressure cooker) antigen retrieval<br>1:40 mouse monoclonal antibody, clone 16P04<br>Horseradish peroxidase (HRP)-labeled polymer conjugated with secondary antibodies | Positive:<br>Five or more squamous epithelial cells with staining of the nucleus, cytoplasm, or both.<br>Positive cases were divided into two semi-quantitative groups:<br>Basal layer staining, in which staining was confined to the basal cells with no staining or very rare staining of more superficial cells;<br>Basal and suprabasal layer staining, defined as positivity of cells in both the basal and suprabasal layers. |

## 1.4 HYPOTHESIS AND AIMS

### Hypothesis

There is some evidence to suggest that HPV may play a role in the development of various cancers and in some cases HPV positivity has a favourable impact on survival. HPV may have a role to play in HNSCC, and more specifically oral carcinoma, and may be of prognostic use. In addition the presence of HPV may identify those dysplastic lesions with a greater potential to progress to OSCC. $p16^{INK4a}$ has been used as a surrogate marker of HPV due to its correlation with HPV DNA but is this justified?

### Aims

The aims of this study are:

- to investigate the incidence of HPV DNA in oral mucosal lesions by polymerase chain reaction
- to compare the incidence of HPV DNA in benign oral lesions; dysplasias; and carcinomas to determine whether there is an association with disease progression
- to utilise a novel sensitive technique to investigate the genotypes of HPV, both alpha and beta HPV, in a subset of oral cancer
- to investigate whether HPV is of prognostic use in oral cancer
- to investigate the expression of p16 in oral tissues
- to investigate the use of p16 as a surrogate marker of HPV DNA in oral tissues
- to investigate the prognostic use of p16 in oral cancer

Figure 1.6 shows the overall strategies to achieve the aims of this study.

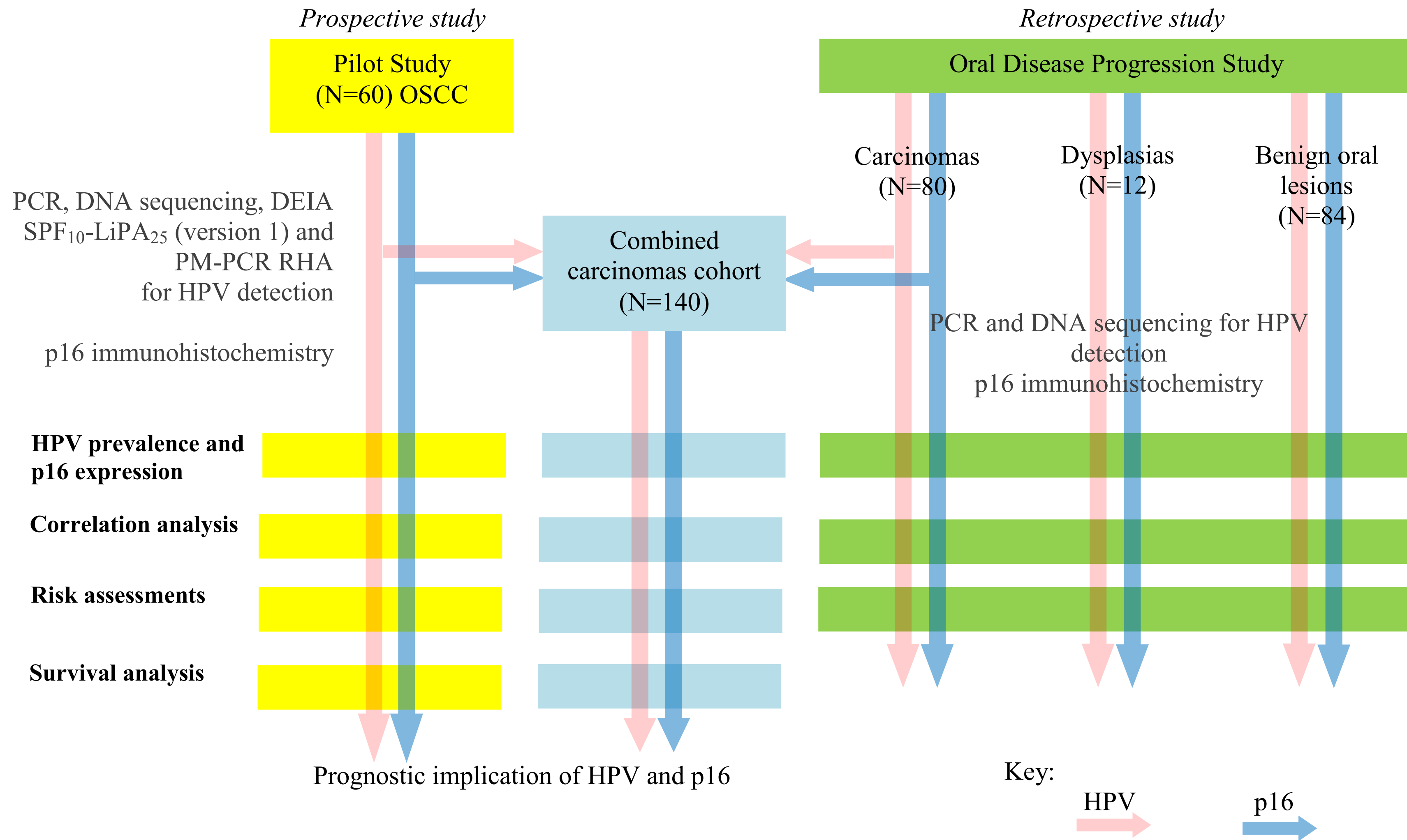



**Figure 1.6: The overall strategies of the study.**
The above approaches will be described further in the appropriate chapter.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 THE ROLE OF HPVs IN ORAL CANCER

### 2.1.1 Introduction

The involvement of certain types of HPV in lesions is clearly understood. The HPVs that have been termed as 'low-risk' (HPV types 6, 11, 13 and 32) are associated with benign lesions of the oral mucosa (Snijders et al., 1996). In contrast, 'high risk' HPV types including HPVs 16, 18, 31, 35 and 39 are associated with premalignant lesions and squamous cell carcinomas (Zur Hausen, 1991; Snijders et al., 1996; Androphy, 1994). PCR-based approaches are now widely used in human papillomavirus (HPV) studies. These highly sensitive approaches are capable of amplifying as low as 10–100 copies of the HPV genome and may give an advantage over other approaches. In addition, only a small amount of specimen is required, and sample preparation prior to amplification is simpler, especially if automation is utilised.

Using this approach, several universal primer sets such as MY11/MY09 (Manos et al., 1989), MY11/GP6 (Manos et al., 1989; Snijners et al., 1990) and GP5+/GP6+ (de Roda Husman et al., 1995) are widely used for routine diagnosis of HPV infection. A novel type-specific six PCR primers designated as Short PCR Fragment (SPF), was developed targeting a 65 bp of HPV L1 region. This SPF system is highly sensitive and capable of detecting at least 43 different HPV genotypes by using a mixture of nine

probes in a microtiter hybridisation format, DNA enzyme immunoassay (DEIA) (Kleter et al., 1998). Recently, a rapid and broad-spectrum PCR amplification was developed utilising 10 SPF primers denoted as $SPF_{10}$ (six novel type specific primer sets and four universal primer sets, MY11/MY09 and GP5+/GP6+). SPF amplimers were detected via reverse hybridisation technique in strip format utilising a 28 line probes assay (LiPA). The combination of the former approach in generating broad-spectrum PCR amplimers and later detection assay, designated as $SPF_{10}$-LiPA simultaneously identified 25 HPV genotypes (Quint et al., 1999).

Another novel one-step PCR (PM-PCR) plus RHA was developed for detection of beta-HPV (PM-PCR RHA) focusing on the E1 region. It was concluded the approach was highly sensitive, reproducible and reliable in dealing with either fresh or FFPETs (de Koning et al., 2006).

### 2.1.2 Aim

The aim of this study was to investigate the expression of human papillomavirus (HPV) in 60 FFPET oral squamous cell carcinomas.

This involved:

- Optimising the DNA extraction from FFPET by a comparison of two different techniques (Promega and Qiagen) using DNA purity and yield as the criteria for assessment.
- Investigating the effect of volume of FFPET on DNA extraction with both methods.

- Using the result of the above to extract DNA from 60 oral cancer samples for analysis by PCR for mucosal alpha HPV L1 gene.
- Using the SPF-DEIA and $SPF_{10}$-LiPA methods [Mucosal (alpha) HPV prototype research assay; DDL, The Netherlands] to identify alpha HPV in the same 60 FFPETs.
- Using the PM-PCR RHA method [Skin (beta) HPV prototype research assay; Diassay BV, The Netherlands] to identify beta HPV in the same 60 FFPETs.

### 2.1.3 General Equipment

*Photographs of selected major instruments used are as shown in Figure 2.1*

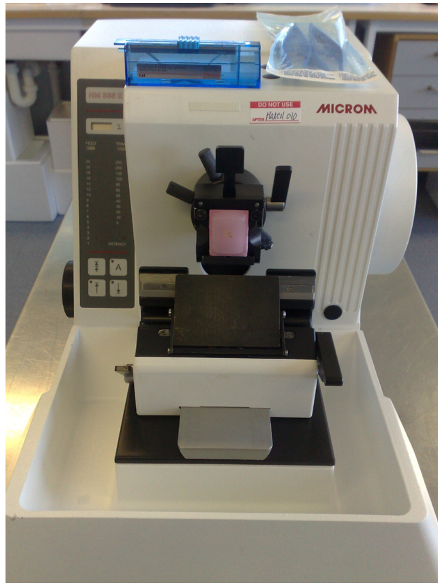
An automated microtome, Shandon Citadel 2000, Microm HM335E

A microtome blades MB 34$^{o}$/80cm (Cat. no. 0022403), Shandon Scientific Limited, Runcorn, Cheshire, England

Forceps or tweezers

Gilson micropipettes (2, 10, 200 and 1000 μl) and filtered (aerosol resistant) micropipette tips (10, 200 and 1000 μl), Rainein Instrument Co. Inc. UK.

Sub Aqua 26 water bath with lid, Grant Instruments (Cambridge) Ltd.




Automated microtome

Whirlimixer

WPA UV1101 Spectrophotometer

Class I Microbiological Safety Cabinet

Benchtop autoclave

Sub Aqua 26 water bath

**Figure 2.1: General instruments**

WPA UV 1101 UV Photometer (80-3000-74), Biochrom Ltd., Cambridge, UK

Quartz cuvettes (80-3000-81), Biochrom Ltd., Cambridge, UK

Class I Microbiological Safety Cabinet, Howorth Air Engineering, Faraworth, Bolton, England

Mettler PM200 topload balance (American Instrument Exchange, Inc.)

Progene thermocycler, 20-sample for PCR (Techne, Gmbh, Germany)

TC-412 thermocycler, 96-well format for PCR (Techne, Gmbh, Germany)

### 2.1.4 Specimens

#### 2.1.4.1 Positive controls (*n*=2+3)

Five positive controls were provided for PCR and HPV genotyping (Appendix 2), 3T3 embryonic mouse fibroblasts (from EACC, Sigma-Aldrich), human salivary glands containing HPV18 DNA and plasmid DNAs containing HPV genes (HPV6, HPV8 and HPV16). The first two (3T3 and HPV18) were positive controls for DNA extraction. The second three positive controls (HPV6, HPV8 and HPV16 DNA) were diluted at the concentration of 10 ηg/µl prior PCR and HPV genotyping.

#### 2.1.4.2 Negative controls (*n*=2)

Two negative controls, tissue free paraffin sections and RNase free water were utilised for PCR and HPV genotyping.

#### 2.1.4.3 Pilot study

Eleven oral FFPET blocks [Appendix 7, samples C1-C6; Appendix 8 and 9, samples D1-D5] were randomly selected for DNA extraction, were obtained from the archives of the Oral Pathology Department, University of Dundee. This was part of the optimisation and the DNA extraction kit comparison. The next sixty oral FFPET blocks were strictly selected based on histopathological and microscopic observations made by pathologists via a double-blind method. The details of the samples used and the patient clinical data in each experiment are given in the appropriate chapter.

#### 2.1.4.4 The role of HPVs in oral disease progression (*n*=183)

In total one hundred and ninety four FFPETs were used in the experiments. 183 oral FFPETs used in the experiments were obtained from Oral Pathology Department, University of Dundee archives. The details of the samples used and the patient clinical data in each experiment are given in the appropriate chapter.

### 2.1.5 Materials for MagneSil® Genomic, Fixed Tissue System, Cat. No. MD1490 (Promega Corporation, USA)

*Kit contents*

MD1170: MagneSil® Genomic, Fixed Tissue Processing Module

- Incubation Buffer, 35 ml
- Proteinase K, 2x 10 mg
- 1M DDT, 1.125 ml

MD1180: MagneSil® Genomic, Fixed Tissue Purification Module

Resin, 0.9 ml

Lysis Buffer, 40 ml

2X Wash Buffer, 30 ml

Elution Buffer, 15 ml

MagneSphere Technology Magnetic Separation Stand (two-position) (Cat.#Z5332)

*Equipments and reagents to be supplied by the user*

95-100% ethanol

Isopropyl alcohol (P/7490/17), Mackay and Lynn (DD) Ltd, Dundee UK

Oven, 56$^{o}$C

Water bath, 65$^{o}$C

Whirlimixer, Fisons Scientific Apparatus, Leicestershire

Microcentrifuge tubes, 1.5 ml (Cat. #V1231)

Aerosol-resistant micropipette tips

### 2.1.6 Materials for QIAamp DNA FFPE Tissue Kit, Catalog no. 56404 (QIAGEN GmbH, Hilden, Germany)

*Kit Contents*

QIAamp MinElute® Columns

Collection tubes (2 ml)

Buffer ATL

Buffer AL (contains guanidine hydrochloride)

Buffer AW1 (concentrate, contains guanidine hydrochloride)

Buffer AW2 (concentrate, contains sodium azide as preservative)

Buffer ATE (contains sodium azide as a preservative)

Proteinase K

*Equipment and Reagents to be supplied by the user*

Xylene, VWR International Ltd, Lutterworth, UK

Ethanol (96-100%), Chemistry Store, University of Dundee

1.5 ml or 2 ml microcentrifuge tubes (for lysis steps)

1.5 ml microcentrifuge tubes (for elution steps) from Brinkmann (Safe-Lock, cat. no. 022363204)

Micropipettes, 10 μl, 20 μl, 200 μl and 1000 μl (BioRad)

Pipette tips, 10 μl, 250 μl and 1000 μl (with aerosol to avoid cross-contamination)

Oven, 56$^{o}$C

Water bath, 90$^{o}$C

Floater

Whirlimixer, Fisons Scientific Apparatus, Leicestershire

Eppendorf centrifuge 5417C, VWR LabShop, Batavia IL

### 2.1.7 Reagents for Polymerase Chain Reaction (PCR)

Photographs of selected major instruments used for this method are as shown in Figure 2.2. The preparation for each reagent is illustrated in Appendix 1 and the components of the PCR as shown in Table 2.1 and 2.2 (except for SPF-DEIA). The final PCR reaction volume for SPF-DEIA was performed at 100 μl. The PCR reaction mixture including 10 μl of 10 ηg/μl of the isolated DNA, PCR buffer [which consists of 10 mmol/L TrisHCl, pH 9.0, 50 mmol/L KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatin], 2.5 mmol/L

$MgCl_2$, 200 mmol/L each deoxynucleoside triphosphate, 20 pmol of six biotinylated PCR primers, of which the mixture of type-specific PCR primers for HPV genotypes (four SPF1 and two SPF2), and 0.25 U of SuperTaq (Sphaero Q, Cambridge, UK) (Kleter et al., 1998). Finally, the PCR conditions for SPF-DEIA are as shown in Table 2.3.

### 2.1.8 Materials for gel electrophoresis

*Photographs of selected instruments used for this method are as shown in Figure 2.3*

Agarose, For Routine Use (Cat. no. A9539-250G), Sigma-Aldrich™ Inc., USA

Agarose, low melting point (Cat. no.A9414-100G), Sigma-Aldrich™ Inc., USA

10X TAE (Tris-acetate/EDTA - ethylenediaminetetraacetic acid)

Loading Dye, Research Biolab Ltd.

100 bp DNA marker, Research Biolab Ltd.

Gel Staining solution Fast Blast DNA Stain (Cat. no. 166-0420EDU), Bio-Rad Laboratories, Inc., USA

Staining container

500 ml conical flask

Weighing boats

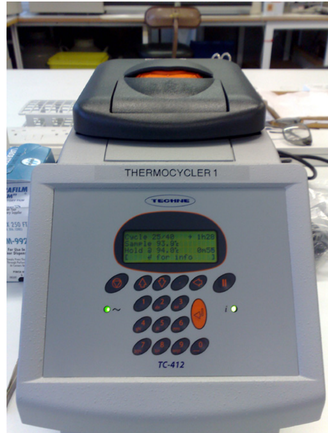
Spatula

Parafilm 50mm wide (SE165-15), Mackay and Lynn (DD) Ltd, Dundee UK

EV243 Electrophoresis power supply (286-026 EV243U), Jencons (Scientific) Ltd., England

Midi horizontal electrophoresis unit (286-312 J-HU13), Jencons (Scientific) Ltd., England

Gel cast and combs (20-well, 16-well and 10-well)




TC-412 thermocycler

Progene thermocycler

8-strip PCR tube with improvised holder

Peltier thermal cycler

**Figure 2.2: Instruments for PCR and RHA**

## Table 2.1: Oligonucleotide primers used for HPV detection

| Human genome | | ORF | Primer | Sequence (5'-3') | Annealing site | | Size (bp) | Degeneracy | Remark |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Bases | | | |
| β-globin | | | GH20<br>PC04 | GAAGAGCCAAGGACAGGTAC[1]<br>CAACTTCATCCACGTTCACC[1] | | 70400-70419<br>70648-70669 | 268 | | Human genomic DNA quality |
| **HPV class** | **HPV group (type) detected** | **ORF** | **Primer** | **Sequence (5'-3')** | **Annealing site** | | **Size (bp)** | **Degeneracy** | **Remark** |
| | | | | | **HPV** | **Bases** | | | |
| Mucosal | General mucosal | L1 | MY11<br>MY09 | GCMCAGGGWCATAAYAATGG[2]<br>CGTCCMARRGGAWACTGATC[2] | 6 | 6722-6742<br>7150-7170 | 450 | 16<br>16 | Mucosal HPV |
| Mucosal | Semi-nested | L1 | MY11<br>GP6 | GCMCAGGGWCATAAYAATGG[2]<br>GAAAAATAAACTGTAAATCA[4] | 6 | 6722-6742<br>6882-6903 | 190 | 16<br>0 | Mucosal HPV |
| Mucosal | Nested | L1 | GP5+<br>GP6+ | TTTGTTACTGTGGTAGATACTAC[3]<br>GAAAAATAAACTGTAAATCATATTC[3] | 6 | 6765-6784<br>6876-6903 | 140 | | Detection for uncommon HPV |
| Mucosal | 43 Alpha-HPV | L1 | SPF1/2 primer mix[5] | Commercially unavailable | 6,11,13,16,18,26,30, 31,33,34,35,39,40, 42,43,44,45,51,52, 53,54,55,56,58,59, 61,62,64,66,67,68, 69,70,72,73,74, MM4,MM7,MM8 | 6582-6646 | 65 | | Mucosal HPV $SPF_{10}$-LiPA[5] [Mucosal (alpha) HPV] |
| Cutaneous | 25 Beta-HPV | E1 | PM Primer mix[6] | Commercially unavailable | 5,8,9,12,14,15,17, 19,20,21,22,23,24, 25,36,37,38,47,49, 75,76,80,92,93,96 | 2644-2760 | 117 | | Cutaneous HPV PM-PCR RHA[6] [Skin (beta) HPV] |

Notes:
*1* Bauer et al., (1991); *JAMA* **265**(4): 472-2
*2* Manos et al., (1989); *Cancer Cells* **7**: 209-214
*3* de Roda Husman et al., (1995); *J Gen Virol* **76**: 1057-62
*4* Snijders et al., (1990); *J Gen Virol* **71**: 173-181
*5* Van Doorn et al., (2006); *J Clin Microbiol* **44**(9): 3292-3298
*6* de Koning et al., (2006); *J Clin Microbiol* **44**(5): 1792-1800

## Table 2.2: Reagents and their composition for each PCR amplification

| PCR amplification | PCR mastermix | | | | | | | | | | | | Template DNA, 10 ng/ul | TOTAL |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Buffer II 10x | $MgCl_2$ (25 mM) | | dNTPs (2.5 mM) | | Primers | | | Amplitaq Gold (5U/µl) | | Distilled water | Total master mix | | |
| | (µl) | (µl) | mM[a] | (µl) | µM[a] | Primer name | (µl) | ρmole | (µl) | Unit | (µl) | (µl) | (µl) | (µl) |
| β-globin gene detection | 5.0 | 3.0 | 1.5 | 4.0 | 200 | PC04 (6 ρmole/µl)<br>GH20 (6 ρmole/µl) | 2.0<br>2.0 | 12<br>12 | 0.2 | 1.0 | 23.8 | 40.0 | 10.0 | 50.0 |
| General PCR for L1 gene of mucosal HPV detection (First round) | 5.0 | 3.0 | 1.5 | 4.0 | 200 | MY11 (50 ρmole/µl)<br>MY09 (50 ρmole/µl) | 1.0<br>1.0 | 50<br>50 | 0.2 | 1.0 | 25.8 | 40.0 | 10.0 | 50.0 |
| Semi-nested PCR for L1 gene of mucosal HPV detection (Second round) | 5.0 | 3.0 | 1.5 | 4.0 | 200 | MY11 (50 ρmole/µl)<br>GP6 (50 ρmole/µl) | 1.0<br>1.0 | 50<br>50 | 0.2 | 1.0 | 32.8 | 47.0 | 3.0[b] | 50.0 |
| Nested PCR for L1 gene of mucosal HPV detection (Second round) | 5.0 | 7.0 | 3.5 | 4.0 | 200 | GP5+ (50 ρmole/µl)<br>GP6+ (50 ρmole/µl) | 1.0<br>1.0 | 50<br>50 | 0.2 | 1.0 | 32.8 | 47.0 | 3.0[b] | 50.0 |
| $SPF_{10}$-LiPA method [Mucosal (alpha) HPV] prototype research assay | 5.0[c] | 5.0 | 2.5 | 4.0 | 200 | Type-specific SPF1/2 and universal primer mix (15 ρmole/µl)[d] | 1.0 | 15 | 0.3 | 1.5 | 24.7 | 40.0 | 10.0 | 50.0 |
| PM-PCR RHA method [Skin (beta) HPV; Diassay BV] prototype research assay | 5.0 | 5.0 | 2.5 | 4.0 | 200 | PM Primer mix[e]<br>(in RHA kit) | 10.0 | | 0.3 | 1.5 | 15.7 | 40.0 | 10.0 | 50.0 |

Notes:

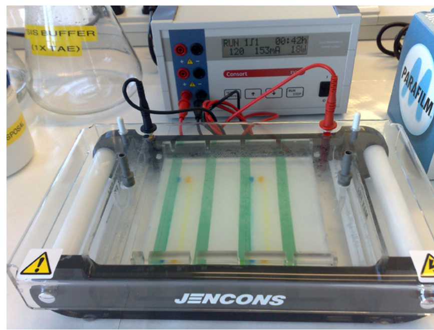
*a* Final concentration; *b* Initial concentration of template DNA used will depend on the PCR product from the first amplification
*c* 10 mmol/L TrisHCl, pH 9.0, 50 mmol/L KCl, Triton X-100, 0.01% gelatin; *d & e* Biotinylated PCR primers
3T3 cells DNA (details preparation be given in Appendix 3) - Positive control for β-globin gene detection
Human Salivary Gland DNA (details preparation be given in Appendix 3) – Positive control for L1 gene of mucosal HPV detection via semi-nested, nested and each independent PCR
HPV type 6 DNA – Positive control for L1 gene of mucosal HPV detection via semi-nested or nested or independent PCR
HPV type 16 DNA - Positive control for L1 gene of mucosal HPV detection via semi-nested or nested PCR, SPF-DEIA and $SPF_{10}$-LiPA method
HPV type 8 DNA - Positive control for PM-PCR RHA method
Negative control 1 – distilled water
Negative control 2 – paraffin alone, which tissue free from FFPET (paraffin to replace tissue during DNA extraction)
Negative control 3 – reagents control

**Table 2.3: PCR condition for each amplification method**

| | Initial Denaturation | | Number of cycles | Final extension | Incubation |
|---|---|---|---|---|---|
| β-globin gene detection | 94ºC for 7 min | Denaturation: 94ºC for 1 min<br>Primer annealing: 55ºC for 1 min<br>Elongation: 72ºC for 1 min | 40 | 72ºC for 7 min | 4ºC |
| PCR for L1 gene of general mucosal HPV detection (First Round) | 95ºC for 10 min | Denaturation: 95ºC for 1 min<br>Primer annealing: 55ºC for 1 min<br>Elongation: 72ºC for 1 min | 30 | 72ºC for 5 min | 4ºC |
| Semi-nested PCR for L1 gene of mucosal HPV detection (Second Round) | 95ºC for 10 min | Denaturation: 95ºC for 1 min<br>Primer annealing: 50ºC for 1.5 min<br>Elongation: 72ºC for 2 min | 30 | 72ºC for 5 min | 4ºC |
| Nested PCR for L1 gene of mucosal HPV detection (Second Round) | 94ºC for 4 min | Denaturation: 94ºC for 1 min<br>Primer annealing: 48ºC for 2 min<br>Elongation: 72ºC for 1.5 min | 40 | 72ºC for 4 min | 4ºC |
| SPF-DEIA [Mucosal (alpha) HPV] prototype research assay[a] | 94ºC for 1 min | Denaturation: 94ºC for 1 min<br>Primer annealing: 45ºC for 1 min<br>Elongation: 72ºC for 1 min | 40 | 72ºC for 5 min | 4ºC |
| $SPF_{10}$-LiPA [Mucosal (alpha) HPV] prototype research assay[b] | 94ºC for 9 min | Denaturation: 94ºC for 30 sec<br>Primer annealing: 52ºC for 45 sec<br>Elongation: 72ºC for 45 sec | 40 | 72ºC for 5 min | 4ºC |
| PM-PCR RHA method [Skin (beta) HPV; Diassay BV] prototype research assay[c] | 94ºC for 9 min | Denaturation: 94ºC for 30 sec<br>Primer annealing: 52ºC for 45 sec<br>Elongation: 72ºC for 45 sec | 35 | 72ºC for 5 min | 4ºC |

Notes:

*a* Kleter et al., (1998); *Am J Pathol* **153**(6): 1731-1
*b* Kleter et al., (1999); *J Clin Microbiol* **37**(8): 2508-2517
*c* de Koning et al., (2006); *J Clin Microbiol* **44**(5): 1792-1800




Horizontal gel electrophoresis set and power pack

3D Rocking platform

Gene Flash, Syngene bioimager

Microwave

Sony gel image printer

Digital camera Nikon D70

**Figure 2.3: Instruments for gel electrophoresis**

A microwave, Technolec T250t: 750 Watts

A 3D Rocking platform, Stuart Scientific STR9

Gene Flash, Syngene Bioimager (Beacon House, Nuffield Road, Cambridge)

Sony Video Graphic Printer, UP-895MD, Tokyo, Japan

### 2.1.9 Materials for PCR Purification Kits

*QIAquick PCR Purification Kits (Cat. no. 28104)*

QIAquick Spin Columns

Buffer PBI – contains chaotropic salt

Buffer PE (concentrate)

Buffer EB

Collection tubes (2 ml)

Loading dye

*QIAquick Gel Extraction Kits (Cat. no. 28704)*

Similar contents as Cat. no. 28104 except Buffer QG to replace Buffer PBI

*For all protocols*

Ethanol (96-100%)

Microcentrifuge

1.5 or 2 ml microcentrifuge tubes

3M sodium acetate, pH 5.0

Distilled water or TE buffer (10 mM Tris-Cl 1 mM EDTA, pH 8) (optional)

*Gel extraction protocol*

Isopropanol (100%)

Water bath

### 2.1.10 Materials for automated PCR products sequencing

*Photographs of selected instruments used for this method are as shown in Figure 2.4*

*Kit Contents*

The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, PN 4337455)

Ready Reaction Mix

BigDye® Terminator v3.1 Sequencing Buffer (5X)

pGEM® -3Zf(+) plasmid, double-stranded DNA Control Template

-21 M13 forward primer (Control)

*Materials supplied by the user*

For cycle sequencing:

GeneAmp® PCR System 9700 (Applied Biosystems)

MicroAmp® 96-Well Reaction Plate (Applied Biosystems, PN N801-0560)

ABI PRISM® Optical Adhesive Cover (Applied Biosystems, PN 4311971)

Sorvall® Legend RT, DJB Labcare Ltd., England

Eppendorf MixMate PCR 96, (Cat. no. EF7505A), Daigger & Company Inc.

For purifying extension products (Ethanol/EDTA precipitation):

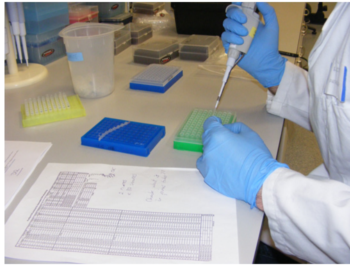
Ethanol (EtOH), 200 proof, Molecular Biology grade

EDTA, 125 mM

Sealing tape

For electrophoresis:

ABI PRISM 3730 DNA Analyser with Data Collection v2.0

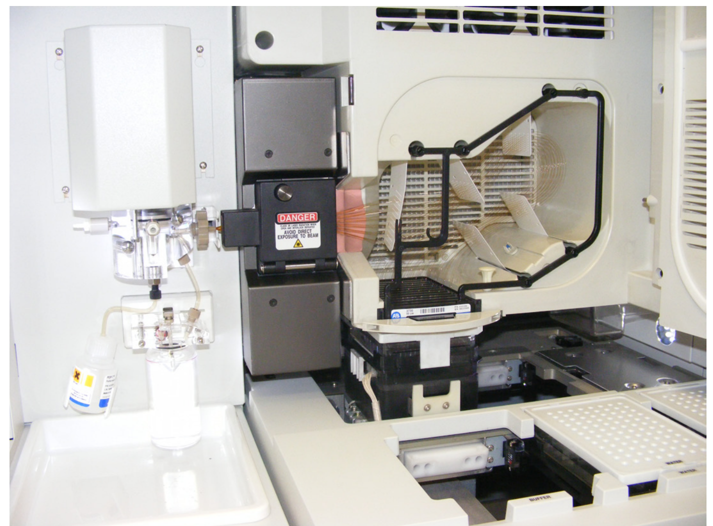


Cycle sequencing reaction
in microtiter plate format

Sorvall microtiter plate centrifuge

Vortex mixer for
microtiter plate

PCR system 9700




3730 DNA Analyser equipped with 48-capillary

**Figure 2.4: Instruments for automated DNA sequencing**

Hi-Di™ Formamide, 25-mL bottle (Applied Biosystems, PN 4311320)

3730 BigDye® Terminator v3.1 Sequencing Standard (Applied Biosystems, PN 4336943)

3700 BigDye Terminator v3.1 Matrix Standard for Spectral Calibration and Dye Set H (Applied Biosystems, PN 4336975)

### 2.1.11 Materials for SPF-DEIA

*Reagents for PCR amplification (as was previously described in section 2.1.7)*

*Materials for Reverse Hybridisation*

Hybridisation buffer (150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0, 0.1% Tween 20)

Microtiter plate streptavidin coated

Denaturation solution (100 mmol/L NaOH)

Digoxigenin (DIG)-labeled HPV-specific probes solution

Anti-DIG alkaline phosphatase conjugate

Substrate solution (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium)

Stopping solution (0.5 mmol/L sulphuric acid)

Microtiter plate reader

### 2.1.12 Materials for $SPF_{10}$-$LiPA_{25}$ (version 1)

*Reagents for PCR amplification (as stated in Table 2.2)*

*Materials for Reverse Hybridisation*

Denaturation solution (NaOH)

Hybridisation buffer (3X SSC [1X SSC is 15 mM Na-citrate and 150 mM NaCl], 0.1% sodium dodecyl sulfate)

Immobilised 28-parallel-line probes on nitrocellulose membrane strips

Fixed 25-trough on tray

Forcep and pencil

Alkaline phosphatase-streptavidin conjugate

Rinse solution

Substrate buffer

Substrate solution (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium)

Stopping solution (distilled water)

Auto-LiPA

### 2.1.13 Materials for Reverse Hybridisation Assay (RHA)

*Photographs of selected instruments used for this method are as shown in Figure 2.2*

*Reagents for PCR amplification (as stated in Table 2.1)*

*Materials for Reverse Hybridisation*

Thin wall with dome shaped PCR microcentrifuge tubes (200 µl)

Removable 8-trough on tray

Immobilised 27-parallel-line probes on nitrocellulose membrane strips

Denaturation solution

3B Buffer

Hybridisation Buffer (3X SSC [1X SSC is 15 mM Na citrate and 150 mM NaCl], 0.1% sodium dodecyl sulphate)

Stringent Wash solution

Alkaline phosphatase-streptavidin conjugate solution (100X concentration)

Conjugate diluent

Rinse solution (5X concentration)

Substrate Buffer

Substrate solution (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) (100X concentration)

### 2.1.14 α and β-HPV typing algorithm for pilot studies

Overall strategies through the pilot studies are summarised in Figure 2.5. Details of each approach will be explained further in the later part of this chapter. All selected FFPET samples were previously mentioned in section 2.1.4. DNA was extracted from the FFPET and quantified prior to testing the quality of the DNA by amplification for β-globin gene.

Three major independent approaches, but inter-related amongst them in HPV genotyping routes were as follows, PCR (semi-nested, nested, and independent), α-HPV detection (via SPF-DEIA and $SPF_{10}$-LiPA) and β-HPV detection utilising RHA method. Finally, confirmation of the HPV types could be made using DNA sequence analysis using BLAST (Basic Local Alignment Systematic Tools) from the Genebank.

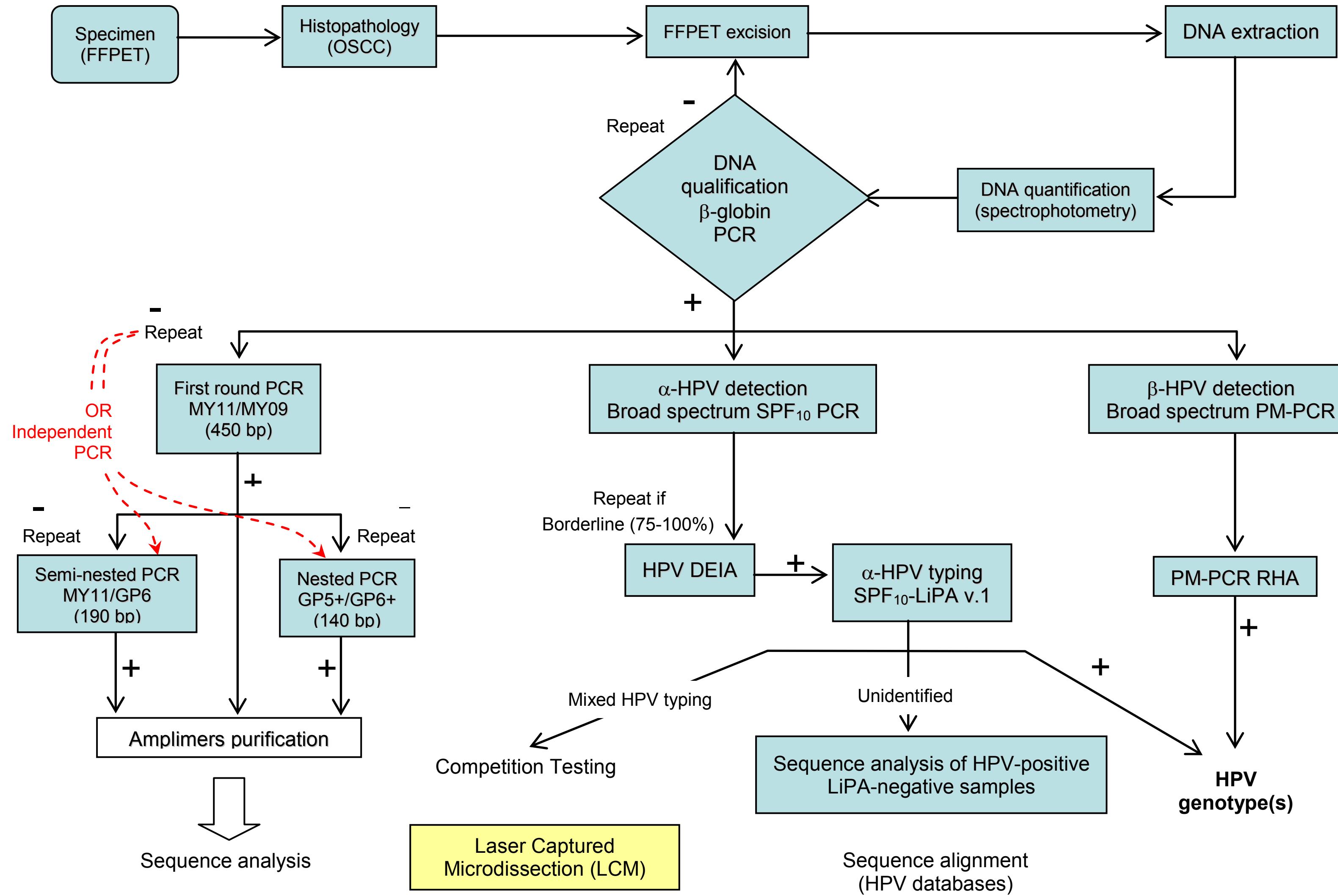



**Figure 2.5: α-HPV and β-HPV detection and genotyping algorithm**

### 2.1.15 Tissue specimen preparation

In order to investigate the effect of tissue volume on DNA yield and purity three volumes of FFPET were studied. The effect of three, five and ten 5 μm sections was studied in triplicate. A spectrophotometer was used to determine the yield and purity of the DNA. The method of DNA quantification and qualification will be described in the appropriate section. The results of this then guided the technique used on the pilot study (*n*= 60) and oral disease progression study (*n*=183). The DNA extraction for the pilot study was carried out in duplicate. In contrast, the DNA extraction for 183 FFPET specimens were carried out once for each sample. The positive and negative controls are as described in 2.1.4.

A microtome was utilised to excise 5 μm sections from each FFPET block for each DNA extraction kit. The first 2-3 sections were discarded to avoid any contamination from air. Tweezers or forceps were used to collect the sections which were immediately placed in a clean 1.5 ml eppendorf tubes. The cutting area on the microtome, especially where the blade was placed and the platform, was carefully wiped with alcohol inbetween tissue blocks. A brand new blade and forceps was also used for the tissue from the subsequent FFPET block to get rid of any DNA contamination from the previous specimen.

### 2.1.16 Isolating DNA from cells and tissues

The key protocols of DNA extraction and purification from cells and tissues are sample lysis and separation of nucleic acid from contaminants. Initially, detergents are used to

solubilise the cell membranes during lysis step. The most common detergents are sodium dodecyl sulphate (SDS), Triton-X and hexadecyltrimethyl ammonium bromide (CTAB). In addition, the combination of a gentle enzyme such as proteinase K and SDS are often used to lyse the cells. This is then followed by treatment with potassium acetate, a cosmotrope to decrease the solubility of protein, SDS and lipid. A white precipitate is usually formed which can be removed via centrifugation retaining the nucleic acid in solution.

Another approach to isolate nucleic acid is to use the combination of chaotropic guanidinium salts and reducing agents (β-mercaptoethanol, dithiothreitol). A chaotrope increases the solubility of molecules and the reducing agent to protect the nucleic acid from oxidative damage. Lysis buffer composition is the major difference when using commercial products and protocols for nucleic acid isolation. The nucleic acid purification is carried out by creating hydrophobic adsorption to silica resin (a solid, predispensed matrix in spin column format) in the presence of chaotropic substances. This can be followed by low salt/ethanol wash to eliminate excess chaotrope and any impurities that present in nucleic acid-resin binding matrix. Finally, nucleic acids are eluted from the silica resin in Tris-EDTA buffer or deionised distilled water and are ready for DNA quantification and use.

### 2.1.17 Human Genomic DNA Extraction from FFPET using a Promega kit

The oven must first be set at 56$^{o}$C before the sample is incubated overnight. Prior to genomic DNA extraction one should ensure that all buffers in the extraction kit are equilibrated to ambient temperature. The water bath must be set at 65$^{o}$C for DNA

elution step. Lysis Buffer ATL needs to be pre-warmed to 37-60°C to dissolve any precipitate, if present. The reagents need to be freshly prepared according to manufacturer's instructions.

100 µl of freshly prepared incubation buffer/proteinase K solution was added to the sections and incubated at 56°C overnight in the oven to deparaffinise the tissues. The tube was removed and 2 volumes of Lysis Buffer added. 7 µl of resin slurry was later added and the mixtures were vortexed for 3 sec at top speed and incubated for 5 min at ambient temperature.

The tube was vortexed for 2 sec at top speed and was placed in the Magnetic Stand for instant separation to occur. The solution in the tube was removed by pipetting taking care not to interrupt the Resin on the other side of the tube. 100 µl Lysis Buffer was added and the tube was removed from the Magnetic Stand and was vortexed for 2 sec at top speed. The tube was returned to the Magnetic Stand and all Lysis Buffer was discarded by pipetting.

100 µl of prepared 1X Wash Buffer was added and the tube was removed from the Magnetic Stand and was vortexed for 2 sec at room temperature. The tube was returned to the Magnetic Stand and all Wash Buffer was carefully discarded by pipetting. The washing steps were repeated at least three times with the prepared 1X Wash Buffer and all of the solution was completely removed for each wash.

The tube was returned to the Magnetic Stand and the lid was kept open to air-dry the Resin for 5 min at ambient temperature. The lid was closed after 25 µl of Elution

Buffer was added and the tube was vortexed for 2 sec at high speed. The tube was incubated in the preheated water bath at 65$^{o}$C for 5 min. The tube was removed and was vortexed for 2 sec at high speed. The DNA-containing solution was transferred to polypropylene containers to reduce the amount of DNA that binds to the sides.

### 2.1.18 Human Genomic DNA Extraction from FFPET using Qiagen kit

Prior to genomic DNA extraction, all buffers in the extraction kit were equilibrated to ambient temperature. The water bath was set at 56$^{o}$C. Buffer AL or Buffer ATL was heated to 70$^{o}$C to dissolve any precipitate, if present. The addition of ethanol to Buffer AW1 and Buffer AW2 was made following manufacturer's instructions prior to being used.

1 ml xylene was added to the sections, the lid was closed and microcentrifuge tube was vortexed vigorously for 10 sec to homogenise the solution. Later centrifugation at full speed (20,000 x g or 14,000 rpm) was carried out for 2 min at room temperature. Supernatant was carefully discarded by pipetting using a fine pipette tips, leaving the pellet untouched at the bottom of the tube.

1 ml ethanol [96-100% (v/v)] was added to the pellet and mixed by vortexing. A similar procedure was followed for centrifugation, removal of any residual ethanol from the specimen. The microcentrifuge tube was opened for 10-minute incubation at room temperature (15-25$^{o}$C) or up to 37$^{o}$C until ethanol had completely evaporated. The pellet was resuspended in 180 μl Buffer ATL and 20 μl proteinase K and homogenised by vortexing. The microcentrifuge tube was incubated at 56$^{o}$C for 1 hour in the water

bath followed by incubation at 90°C for 1 hour in the incubator to completely lyse the specimen. Subsequently, the 1.5 ml tube was briefly centrifuged to eliminate drops from the internal lid.

A premixed solution containing 200 µl Buffer AL and 200 µl ethanol [96-100% (v/v)] was added to the specimen. Each tube was vortexed to obtain a homogeneous solution and briefly centrifuged to remove drops from the inside of the lid. The entire lysate was carefully transferred to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, the lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. QIAamp MinElute column was then placed in a clean 2 ml collection tube and the collection tube containing the flow-through was discarded.

500 µl Buffer AW1 was carefully added to the QIAamp MinElute column without wetting the rim, the lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. QIAamp MinElute column was then placed in a clean 2 ml collection tube and the collection tube containing the flow-through was discarded. 500 µl Buffer AW2 was carefully added to the QIAamp MinElute column without wetting the rim, the lid was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. QIAamp MinElute column was later placed in a clean 2 ml collection tube and the collection tube containing the flow-through was discarded.

Each tube was centrifuged at full speed (20,000 x g or 14,000 rpm) for 3 min to completely dry the membrane. QIAamp MinElute column was then placed in a clean 2 ml collection tube and the collection tube containing the flow-through was discarded. 50 µl of Buffer ATE was carefully applied to the centre of the membrane of the column

without wetting the rim, the lid was closed and incubated at room temperature (15-25$^{o}$C) for 5 min and centrifuged at full speed (20,000 x g or 14,000 rpm) for 1 min.

### 2.1.19 Quantification and Qualification of Extracted DNA

The spectrophotometer, WU 1101 or NanoVue (GE Healthcare UK Ltd.) was first set for measuring nucleic acids and selected for DNA and default settings were recommended for routine measurements. DNA parameters such as pathlength (automatically adjusted either 0.2 or 0.5 mm), to turn off background subtraction and dilution factor with the unit of DNA concentration been given as μg/μl. The filters provided in the instrument automatically read the absorbance at wavelengths (230, 260, 280 and 320 nm) together with the absorbance ratio of 260/280. The ratio range from 1.6 and 2.2 gave an indication the purity of DNA samples.

2 μl of reference solution (DNA elution buffer, Tris-EDTA buffer) was pipetted into the indicated well and the sample head was lowered. The spectrophotometer was zeroed by pressing the reference button to read. Once the measurement was complete, the sample head was raised and the top and bottom plates were cleaned with a lint-free tissue. Similarly, 2 μl of reference solution was pipetted and the reading was repeated. The plates were cleaned and later multiple samples were measured once the reference information was stored. 2 μl of sample was used for testing. The measurement was performed in triplicate and the average for each parameter was recorded without removing the sample.

All the details of these measurements are listed below and will be discussed in the appropriate chapter.

a. The quality and quantity of the positive controls (*n*=2+3) (human salivary glands, 3T3 embryonic mouse fibroblast cells, HPV16/6/8).
b. Repeated measurements variation within and between DNA samples extracted from FFPETs (*n*=6).
c. The effect of DNA volume to the DNA extraction (*n*=5) in triplicate.
d. DNA extraction methods comparison (Qiagen kit versus Promega kit) (*n*=5) in triplicate.
e. Extracted DNA for pilot study using Qiagen kit (*n*=60) in duplicate.
f. Extracted DNA for HPVs role in oral disease progression study using Qiagen kit (*n*=183).

### 2.1.20 Agarose gel electrophoresis

#### 2.1.20.1 Agarose gel preparation

0.2 g of standard agarose was weighed and was poured into 250 ml conical flask. 100 ml of Tris-Acetate EDTA (1X TAE) (*gel buffer*) and was added and mixed evenly. The flask was microwaved until the agarose was fully melted. Periodically the flask was removed from the microwave and gently swirled to homogenise the agarose without making bubbles and ripples. The agarose was left on the bench until its temperature was approximately 50$^{o}$C.

2.1.20.2 Gel Casting

The rubber gaskets were inserted into the groove at both edges of the gel tray. This was to give a good contact between the open edges of the gel tray to the sides of the pouring box and to avoid any leakage prior the agarose being poured into it. The gel tray was pushed down into the pouring box. Alternatively, transparent rubbers were securely attached to both open edges of the gel cast. It was left on an even surface at room temperature.

A comb of appropriate width, size and number was placed into the niche at the end of the tray. The agarose solution was poured evenly into the tray and left for about 15 min to solidify and completely set for another 15 min. The gel tray was lifted and both rubber gaskets were removed. The comb was removed by lifting it straight up. The gel tray was rotated 90$^{o}$ to ensure the ends of the gel were exposed to the ends of the gel box. The well was positioned at the cathode. 400 ml of 1X TAE buffer (*electrophoresis buffer*) was added to fill both the gel tanks and gel was ensured to be completely immersed.

2.1.20.3 DNA sample loading

Gel loading dye, Blue (6X) is a premixed tracking dye with loading buffer for agarose gel electrophoresis. It contains SDS (to obtain sharper DNA bands), EDTA (to chelate magnesium for stopping enzymatic reaction if present) and bromophenol blue (a universal tracking dye for electrophoresis). On a standard agarose gel 1% TBE, it migrates at approximately 300 bp.

1 μl of loading dye was gently mixed with 5 μl of DNA sample by pipetting up and down on parafilm (working concentration of loading dye is 1X). The homogenous mixture of DNA sample and loading dye was gently loaded by pipetting out into the second well of the agarose gel. The first well was reserved for the DNA marker either 100 bp DNA ladder or 1 Kb DNA ladder.

#### 2.1.20.4 Gel electrophoresis

The cover lid of electrophoresis set was closed. The power supply was set for electrophoresis running time of 1 hour at constant voltage of 120V. The power supply was started and the progress of the migrated DNA was monitored regularly by migration of the loading dye. Once the electrophoresis was complete, the power supply was switched off and the agarose gel was removed for staining.

#### 2.1.20.5 Gel staining using Fast Blast DNA Stain

Fast Blast DNA stain is a non-toxic and non-carcinogenic option to ethidium bromide in the agarose gel after electrophoresis to detect the DNA present. The thiazin family of dyes (cationic substance – positively charged) is one of its components. Negatively charged phosphate groups on the DNA molecules are attracted to the dye molecules. The staining procedure generally takes longer than conventional fluorescent DNA stains like ethidium bromide due to the fact that Fast Blast is a non-fluorescent observable stain. The minimum quantity of DNA that can be visualised in an agarose gel either as a quick or an overnight staining using this method is 50 ηg of DNA. The quick staining usually takes around 15-30 min to complete. It was recommended to use 60 ml of 100X

Fast Blast to stain 7 x 7 cm or 7 x 10 cm agarose gel and the stain can be reused at least 7 times. The DNA bands of the stained gel may emerge blurred initially but turned into sharper bands gradually within 5-15 min after the second wash. The preparation of reagents for quick or overnight staining is illustrated in Appendix 34.

The agarose gel was placed in the staining tray after electrophoresis. 100X Fast Blast DNA stain was poured into the tray and the gel was stained for 2-3 min. Used staining solution was poured back into a storage bottle by using a funnel for future use. The gel was transferred into a vessel containing 500-700 ml of warm (40-55$^{o}$C) clean tap water. The gel was gently shaken on a rocking platform for 5 min for washing. The washing procedure was repeated twice.

2.1.20.6 Gel image capturing

The agarose gel was placed on the platform inside the Syngene imager. The gel image (black and white) was captured and printed using an integrated Sony printer. Alternatively, a coloured gel image was generated by using a digital camera, Nikon D70.

### 2.1.21 Optimisation of Polymerase Chain Reaction (PCR)

#### 2.1.21.1 PCR for detection of human β-globin gene

A preliminary effort to optimise the PCR amplification was carried out using the positive and negative controls in triplicate. As a gold standard for quality checking of the extracted DNA from human samples and cells, detection of β-globin gene expression was used. The DNA sequence of human β-globin gene (Appendix 4) and the location of both primer pairs flanking the target region are shown in Figure 2.6. Two positive controls carrying human DNA were tested as mentioned in 2.1.15 and described further in Appendix 3. The initial PCR amplifications as shown in Table 3 and preparation of each reagent is illustrated in Appendix 2. 10 μl of extracted DNA at 10 ηg/μl concentration was added to 40 μl of mastermix as shown in Table 2. 50 μl of reaction mixture for each specimen underwent PCR amplification according to condition shown in Table 2.3.

```
LOCUS       NG_000007              81706 bp    DNA     linear   PRI 25-JAN-2009
DEFINITION  Homo sapiens beta globin region (HBB@); and hemoglobin, beta (HBB);
            and hemoglobin, delta (HBD); and hemoglobin, epsilon 1 (HBE1); and
            hemoglobin, gamma A (HBG1); and hemoglobin, gamma G (HBG2), on
            chromosome 11.
ACCESSION   NG_000007
VERSION     NG_000007.3  GI:28380636
KEYWORDS    RefSeqGene.
SOURCE      Homo sapiens (human)

    ......
                               GH20
    70381 aactcctaag ccagtgccag aagagccaag gacaggtacg gctgtcatca cttagacctc
    70441 accctgtgga gccacaccct agggttggcc aatctactcc caggagcagg gagggcagga
    70501 gccagggctg ggcataaaag tcagggcaga gccatctatt gcttacattt gcttctgaca
    70561 caactgtgtt cactagcaac ctcaaacaga caccatggtg catctgactc ctgaggagaa
    70621 gtctgccgtt actgccctgt ggggcaaggt gaacgtggat gaagttggtg gtgaggccct
                                                 PC04
    ......
```

**Figure 2.6: GH20 and PC04 primer annealing sites in human beta globin gene**

### 2.1.21.2 PCR for detection of protein L1 of mucosal HPV

An initial effort to optimise the PCR amplification and conditions was carried out using a single positive control (a plasmid containing insert of HPV 6 L1 gene) and several negative controls. The strategies and algorithm for detection of the alpha HPV L1 gene expression was carried out either via PCR-gel electrophoresis, SPF-DEIA or $SPF_{10}$-LiPA. The last two approaches will be explained further in section 2.1.25.1 and 2.1.25.2, respectively. Figure 2.7 shows the DNA sequence of HPV 6 L1 protein gene, the location of three universal primer pairs (MY11/MY09, MY11/GP6 and GP5+/GP6+), type-specific SPF primer sets and designed HPV detection probes (shaded DNA sequences) flanking the target region. Each primer set, MY11/MY09, MY11/GP6, GP5+/GP6+ and SPF1/SPF2 was expected to generate approximately 450 bp, 190 bp, 140 bp and 65 bp amplimers, respectively.

```
LOCUS       AF092932                8012 bp    DNA     linear   VRL 14-NOV-2001
DEFINITION  Human papillomavirus type 6, complete genome.
ACCESSION   AF092932
VERSION     AF092932.1  GI:6002612
KEYWORDS    .
SOURCE      Human papillomavirus type 6
  ORGANISM  Human papillomavirus type 6
            Viruses; dsDNA viruses, no RNA stage; Papillomaviridae;
            Alphapapillomavirus.

    ......
    ......
                                                        SPF1
     6541 tgtttgccag acattttttt aacagggctg gcgaggtggg ggaacctgtg cctgatactc
     6601 ttataattaa gggtagtgga aatcgaacgt ctgtagggag tagtatatat gttaacaccc
                                                         SPF2
     6661 caagcggctc tttggtgtcc tctgaggcac aattgtttaa taagccatat tggctacaaa
           MY11                                            GP5+
     6721 aagcccaggg acataacaat ggtatttgtt ggggtaatca actgtttgtt actgtggtag
     6781 ataccacacg cagtaccaac atgacattat gtgcatccgt aactacatct tccacataca
     6841 ccaattctga ttataaagag tacatgcgtc atgtggaaga gtatgattta caatttattt
                                               GP6+    GP6
     6901 ttcaattatg tagcattaca ttgtctgctg aagtaatggc ctatattcac acaatgaatc
     6961 cctctgtttt ggaagactgg aactttgggt tatcgcctcc cccaaatggt acattagaag
     7021 atacctatag gtatgtgcag tcacaggcca ttacctgtca aaagcccact cctgaaaagg
     7081 aaaagccaga tccctataag aaccttagtt tttgggaggt taatttaaaa gaaaagtttt
     7141 ctagtgaatt ggatcagtat cctttgggac gcaagttttt gttacaaagt ggatataggg
                                    MY09
     7201 gacggtcctc tattcgtacc ggtgttaagc gccctgctgt ttccaaagcc tctgctgccc
     7261 ctaaacgtaa gcgcgccaaa accaaaaggt aatatatgtg tatatgtact gttatatata

    ......
    ......
```

**Figure 2.7: Primer annealing sites for universal primer sets and type-specific SPF primer sets and probes in HPV 6 L1 protein**

### 2.1.22 PCR using extracted genomic DNA

#### 2.1.22.1 The detection of β-globin gene (268 bp)

The amplification of a 268 bp β-globin gene demonstrated that the extracted human genome DNA from FFPET was of sufficient quality for further PCR amplifications as shown in Table 2.1. Preparation of each reagent is illustrated in Appendix 2. 10 µl of extracted DNA at 10 ηg/µl concentration was added to 40 µl of mastermix as shown in Table 2.2. 50 µl of reaction mixture for each specimen was undergone PCR amplification according to condition as stated in Table 2.3.

For the pilot study, DNA from 60 FFPET specimens was analysed for quality as mentioned above, in triplicate. Similarly, for larger samples consisting of 183 extracted DNA from three different pathological groups (benign, dysplasia and carcinoma) were to be positively confirmed prior L1 gene HPV detection. Each sample was considered positive for the β-globin gene if it exhibited very clear and convincing data for at least one repetition. Only doubtful positive or negative results were re-analysed either by PCR or the DNA was re-extracted to ensure potentially positive results were never missed. Finally, those samples positively identified at this stage qualified for the next stage which was HPV gene detection and the rest of the samples were identified as negative.

#### 2.1.22.2 Semi-nested PCR for detection of L1 gene of mucosal HPV

The positive samples from 2.1.22.1 were qualified for HPV detection. DNAs from FFPET were suitable for first round PCR amplification of approximately 450 bp expected product size, in triplicate, using MY11/MY09 primer pair as shown in Table 2.1. The related positive and negative controls for PCR were also included. 10 μl of extracted DNA at 10 ηg/μl concentration was added to 40 μl of mastermix as shown in Table 2.2. 50 μl of reaction mixture for each specimen was used for PCR amplification according to conditions stated in Table 2.3. All PCR products from the first round PCR amplification were used for the second round PCR amplification to obtain a 190 bp product using MY11/GP6 primer pair as shown in Table 2.1. 3 μl of product from the first round of PCR was added to 47 μl of mastermix as shown in Table 2.2. 50 μl of reaction mixture for each specimen was amplified by PCR according to conditions stated in Table 2.3.

2.1.22.3 Nested PCR for detection of L1 gene of general mucosal HPV

All PCR products of 450 bp in size from the first round PCR amplifications were used for the second round PCR amplification in triplicate to obtain 140 bp using GP+/GP6+ primer pair as shown in Table 2.1. Positive and negative controls were also included to estimate the performance of PCR and to detect unnecessary DNA contaminants that might be present in the reaction. 10 µl of extracted DNA at 10 ηg/µl concentration was added to 40 µl of mastermix as shown in Table 2.2. 50 µl of reaction mixture for each specimen amplified by PCR according to the conditions stated in Table 2.3.

2.1.22.4 Independent PCR for detection of L1 gene of mucosal HPV using two separate primer pairs

The rational for this direct approach is an alternative route for those DNA samples which failed to produce a 450 bp amplimer from the first PCR amplification. Since dealing with extracted DNA from FFPETs, it is a big challenge to amplify bigger products and this relies upon the moderate quality of the DNA sources.

Independent PCR amplifications were carried out using MY11/GP6 and GP5+/GP6+ primer pairs and fresh DNA template. These were in done in separate reactions to increase the chances of obtaining positive results due to the expected size of both amplimers being relatively small, 190 bp and 140 bp.

### 2.1.23 Purification of PCR products

The QIAquick system was designed for fast DNA purification which includes:

- QIAquick PCR Purification Kit

  This kit is mainly used for robust analysis of PCR products involving double- or single-stranded PCR products within the size range of 100 bp – 10 Kb.
- QIAquick Gel Extraction Kit

  These kits were used to improve DNA recovery and to extract DNA fragments around the size range of 70 bp – 10 Kb from either standard agarose or low-melt agarose gels in TAE (Tris-acetate/EDTA) or TBE (Tris-borate/EDTA) buffer.

#### 2.1.23.1 QIAquick PCR Purification Kit

5 volumes of Buffer PBI were added to 1 volume of the PCR sample and were mixed by pipetting. The colour of the mixture should be yellow (to indicate pH ~ 7.5 which is optimal for DNA binding) after the gel slice has completely dissolved. 10 µl of 3M sodium acetate, pH 5.0 was added to the mixture (if the colour is orange or violet after the incubation) to turn the colour to yellow.

A QIAquick spin column was placed in a 2 ml collection tube. The sample was applied to the column and was centrifuged at 17,900 x g (13,000 rpm) for 30–60 sec. The flow-through was discarded and the column was returned to the same tube. 750 µl of Buffer PE was added to the QIAquick column and was centrifuged at 17,900 x g (13,000 rpm) for 30–60 sec. The flow-through was discarded and the QIAquick column

was returned to the same collection tube and was centrifuged for an additional 1 min at 17,900 x g (13,000 rpm).

The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) was added to the centre of the QIAquick membrane and the column was centrifuged for 1 min. 1 volume of Loading Dye was added to 5 volumes of purified DNA and was mixed by pipetting if the DNA is to be analysed on a gel.

2.1.23.2 QIAquick Gel Extraction Kit

The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The size of the gel slice was reduced by removing extra agarose. The gel slice was weighed in a colourless tube and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg~100 µl). 6 volumes of Buffer QG were added for 2% (w/v) agarose gel and the maximum amount of gel slice per QIAquick column is 400 mg.

The tube was incubated at 50$^{o}$C for 10 min or until the gel had completely dissolved. The colour of the mixture should be yellow (to indicate pH ~ 7.5 which is optimal for DNA binding) after the gel slice has completely dissolved. 10 µl of 3M sodium acetate, pH 5.0 was added to the mixture (if the colour is orange or violet after the incubation) to turn the colour to yellow. 1 gel volume of isopropanol was added to the sample which was then homogenised by inverting the tube 3-5 times.

The QIAquick spin column was placed in a 2 ml collection tube. The sample was applied to the QIAquick column and was centrifuged at 17,900 x g (13,000 rpm) for 1 min. The maximum quantity of the column reservoir was 800 µl. The flow-through was discarded and the QIAquick column was returned to the same collection tube. 500 µl of Buffer QG was added to QIAquick column and was centrifuged at 17,900 x g (13,000 rpm) for 1 min.

The flow-through was discarded and the QIAquick column was returned in the same collection tube. 750 µl of Buffer PE was added to QIAquick column and was centrifuged at 17,900 x g (13,000 rpm) for 1 min. The flow-through was discarded and the QIAquick column was returned in the same collection tube and was centrifuged for an additional 1 min at 17,900 x g (13,000 rpm).

The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) was added to the centre of the QIAquick membrane and the column was centrifuged for 1 min to elute the DNA. 1 volume of Loading Dye was added to 5 volumes of purified DNA which was mixed by pipetting if the DNA is to be analysed on a gel.

2.1.23.3 Quantification and Qualification of the purified PCR products

PCR products purified by using either the former or the latter method were assessed quantitatively and qualitatively by spectrophotometry as described in the section 2.1.19. Statistical analyses were carried out in the appropriate chapter as stated for comparison of their means. The measurements include:

a. All amplicons from pilot study (*n* = 67; all carcinoma); CHAPTER 3
b. All amplicons from oral disease progression study (*n* = 142; which include carcinoma, *n* = 67); CHAPTER 4
c. Carcinoma, *n* = 134 (combination from data *a* and *b*, *n* = 67 + 67); CHAPTER 5

#### 2.1.24 Automated DNA Sequencing of the PCR products

In principle, the purified DNA fragments amplified by PCR are denatured to single strands and one of the strands is hybridised to a primer. Heat-resistant *Taq* polymerase and a pool of deoxyribonucleotide triphosphates (dNTPs) allow the synthesis of new DNA strands from the terminal of primer sequence. This is similar to the PCR method except a single primer is required (either forward or reverse primer) in each reaction and one of four chain-terminating and fluorescent-labelled nucleotides, ddNTPs. For instance, if ddGTP is used, the resulting synthesis of nested DNA fragments in variable sizes and each fragment ends at one of the Gs in the sequence through the replacement of a fluorescent-labeled ddGTP. Similarly, a set of fragments is created for each of the other three bases and is labelled according to ddNTPs being used.

Electrophoresis in a capillary for each sample separates a mixture of all labelled DNA fragments by size. The presence of each of the four labels is determined by scanning the ladder of fragments and a computer programme then analyses the probable order of the peaks and predicts the DNA sequence.

2.1.24.1 Cycle Sequencing

The automated DNA sequencing of amplimers was performed by an experienced technician. The reaction mixtures to be added for each PCR products are as shown in Table 2.4. This preparation was made for MicroAmp® 96-Well Reaction Plate. An ABI PRISM® Optical Adhesive Cover was used to seal the plate to avoid environmental contamination. All the cycle sequencing mixtures in the plate were well mixed using Eppendorf MixMate PCR 96.

**Table 2.4: Reagents for cycle sequencing of PCR products**

| Reagent | Quantity |
|---|---|
| Terminator Ready Reaction Mix<br>Ready Reaction Premix, 2.5X and BigDye® Terminator v3.1 Sequencing Buffer, 5X | 4 μl<br>+<br>2 μl } 5 μl of the mixture was used for each reaction |
| Template DNA[1] (PCR product) | 1-3 ηg (140 bp and 190 bp amplicons)<br>3-10 ηg (450 bp amplicons) |
| Primer[2]<br>Forward or reverse | 5 ρmol |
| Deionised water | Top up to the final volume |
| Final volume | 20 μl |

Notes:
1 pGEM®- 3Zf(+) plasmid was used as a control for cycle sequencing
2 -21 M13 forward primer for control template

Later, the plate was placed in the GeneAmp® PCR System 9700 thermal cycler and the amplification was carried out according to the conditions in Table 2.5. Each extended products were held at 4°C in thermo cycler until ready for purification.

**Table 2.5: Cycle sequencing condition of each sample**

| | | |
|---|---|---|
| Initial Denaturation | 96$^{o}$C for 1 min | - Rapid termal ramp to 96$^{o}$C |
| Denaturation<br>Primer annealing<br>Extension | 96$^{o}$C for 10 sec<br>50$^{o}$C for 5 sec<br>60$^{o}$C for 4 min | - 25 cycles and<br>rapid termal ramp to each<br>setting temperature |
| Incubation | 4$^{o}$C | - Rapid termal ramp to 4$^{o}$C and was held until ready for extension products purification |

2.1.24.2 Purification of the extension products via ethanol/EDTA precipitation method

The 96-well reaction plate was removed from the thermal cycler and was briefly spun down using a Sorvall® Legend RT centrifuge. 5 µl of 125 mM EDTA was added to each well and to ensure the EDTA was completely mixed with the extension products. It was followed by the addition of 60 µl of 100% ethanol to each well. Later, the plate was sealed with optical adhesive cover and the content of each well was mixed by inverting 4 times.

The plate was incubated at room temperature for 15 min and subsequently was spun at the maximum speed of 1400-2000 x *g* for 45 min using Sorvall® Legend RT centrifuge which was set at 4$^{o}$C. The plate was inverted and was spun up to 185 x *g*, and then was removed from the centrifuge. 60 µl of 70% (v/v) ethanol was added to each well and the plate was centrifuge at 4$^{o}$C for 15 min at 1650 x *g*. The plate was inverted and was later spun up to 185 x *g*, and was then removed from the centrifuge.

2.1.24.3 Sample electrophoresis

The samples and standards were resuspended in injection solution, Hi-Di formamide (PN 4311320). The volume of the sample in the reaction plate wells for precise delivery

of samples to the capillary array tips (48-capillary with 36-cm array length for 3730 DNA Analyzer) is illustrated in Table 2.6. The plate was immediately covered by 3100 Genetic Analyzer Plate Septa (PN 4315933) or Optical Heat-Seal Film (PN 4337570) to avoid sample degradation.

**Table 2.6: Resuspension volumes for reaction plates**

| Reaction plate | Minimum volume (ul) | | Maximum volume (ul) | | Recommended volume (ul) |
|---|---|---|---|---|---|
| | Film | Septa | Film | Septa | |
| 96-well | 10 | 10 | 200 | 150 | 10-30 |

The reaction plate was centrifuged at 2000 x *g* for 1 min to bring down the samples to the bottom of the wells and later the reaction plate was carefully examined the position of the samples. The reaction plate was later placed in the integrated plate stacker of 3730 DNA Analyzer (which can accommodate 16 sample plates – 96-well format). The electrokinetic injection, EKI (V-Sec/cm) has to be optimised following parameters: injection time (seconds), injection voltage (Volts/cm) and the concentration of DNA fragments in the sample according to the strength of the signal and the quality of the resolution. The default Standard Run Module was set which was suitable to detect maximum 850 bases. The first 20 min was programmed to run the protocol using POP-7™ polymer and Dye Set H following parameters: the separation voltage was 8.5kV and electrophoresis temperature was 60$^{o}$C. It was followed by data collection (approximately 20 to 30 min for 450 bases) which started after DNA fragments travelling through a 36-cm capillary array and being scanned by fluorescence detector.

2.1.24.4 DNA sequencing outputs

Sequence Analysis software provided with the 3730 DNA Analyzer system gave the results in the form of basecaller/s (further details in Appendix 5). The raw DNA sequence data in which three different and integrated files was created for each sample, chromatogram, SCF chromatogram and SEQ-sequence files. Viewing and final editing of the uncertain or ambiguous bases from the chromatogram file can be made using Chromas Lite 2.0 software (Technelysium Pty Ltd). Manually edited sequences need to be saved and exported into SCF chromatogram and SEQ-sequence files (in FASTA format). Contig for each sample (overlapping and non-overlapping sequence) will be generated by combining forward and reverse primer results (SCF chromatogram files were used) using DNA Baser (http://www.dnabaser.com/, Heracle Software, Germany) or DNA for Windows Ver. 2.2.1 (http://www.dna-software.co.uk/, University of Durham) softwares. The contig tells us the actual size of PCR products being sequenced in base pairs. Subsequently, the generated contig was used to search for HPV DNA sequence similarity using BLAST from the Genebank. Pair-wise and multiple sequence alignments of the contigs can be performed by using stand-alone programme such as ClustalW2 (http://www.ebi.ac.uk/Information/ebi_bac/, European Molecular Biology Laboratory). The former for comparing two sequences and the later for more than two sequences simultaneously.

### 2.1.25 SPF-DEIA and $SPF_{10}$-$LiPA_{25}$ (version 1) for alpha-HPV genotypes

#### 2.1.25.1 SPF-DEIA

Initial PCR amplifications were as described in section 2.1.7 and preparation of each reagent is illustrated in Appendix 2. 10 µl of extracted DNA at 10 ηg/µl concentration was added to 90 µl of mastermix. 100 µl of reaction mixture for each specimen was undergone PCR amplification with positive, borderline positive and several negative controls according to condition as stated in Table 3.

PCR products, generated by six biotinylated primers were detected via reverse hybridisation in a microtiter plate utilising a composition of nine designed probes based on DNA sequences of 39 HPV genotypes at L1 open reading frame region (Kleter et al., 1998). 100 µl of hybridisation buffer was mixed with 10 µl of amplimer in a streptavidin coated microtiter plate well and incubated at 42$^{o}$C for 30 min. Unbound substances were discarded by three washing steps with hybridisation buffer. 100 µl of denaturation solution was added to the captured amplimers and the plate was incubated at ambient temperature for 5 min. Again, it was followed with three washes with hybridisation buffer.

Digoxigenin (DIG)-labeled HPV-specific probes solution was freshly diluted with hybridisation buffer, added to each well and incubated for 45 min at 42$^{o}$C. Anti-DIG alkaline phosphatase conjugate was added to each well and was incubated for 45 min at 42$^{o}$C following three washing steps. Subsequently, substrate was added and followed by incubation at room temperature for 5 min. After, each well was washed for five times.

Finally, the addition of 100 μl of 0.5 mmol/L of sulphuric acid stopped the reaction. A microtiter plate reader was used to measure an optical density (OD) of the content of each well at 450 nm. Positives were identified if the recorded $OD_{450}$ was 2.5 times higher than the negative PCR control. PCR amplification and followed by DEIA detection were repeated if the reading within borderline value (75 to 100% of the cut off value) (Van Doorn et al., 2006).

2.1.25.2 $SPF_{10}$-$LiPA_{25}$ (version 1)

PCR amplifications mentioned in section 2.1.25.1 were performed by using reagents and as shown in Table 2.2 and preparation of each reagent is illustrated in Appendix 2. 10 μl of extracted DNA at 10 ηg/μl concentration was added to 40 μl of mastermix as shown in Table 2.2. 50 μl of reaction mixture for each specimen was amplified by PCR with positive, borderline positive and several negative controls according to conditions stated in Table 2.3.

PCR products, generated by ten biotinylated primers were detected via reverse hybridisation in nitrocellulose membrane strips format and simultaneously identified 25 HPV genotypes. It was denoted as line probe assay (LiPA) utilising a composition of twenty eight designed specific probes based on DNA sequences of 39 HPV genotypes at L1 open reading frame region (Kleter et al., 1999).

Hybridisation solution and Stringent Wash solution must be prewarmed at 37°C and all crystals should be dissolved by mixing prior being used. The LiPA strip was removed from the tube using forceps and was labelled in pencil according to sample

identification number above the line of the strip. Care had to be taken not to touch below the line of the strip with a bare hand to avoid any contamination.

10 μl biotinylated amplimer was pipetted into the upper corner of each labelled trough. Then, 10 μl of NaOH was added and the solutions carefully mixed by pipetting. The denaturation process was performed at room temperature for 10 min. The labelled strip was immediately placed in the labelled test trough (one strip per trough and a total of twenty five troughs per tray). The strips should be facing up as the label at the top.

Thereafter, all incubations and washings were automatically done in Auto-LiPA with pre-set programmes. 2 ml of prewarmed hybridisation buffer was automatically added to each trough and incubated for an hour at $50 \pm 0.5^{o}C$. The removal of the solution was done and followed by stringent wash at $50^{o}C$ with 2 ml of hybridisation solution to each strip, twice for 30 sec and once for 30 min.

Later, the strip was incubated for 30 min at ambient temperature with 2 ml of alkaline phosphatase-streptavidin conjugate. It was followed by washing steps, twice with 2 ml of rinse solution and once with 2 ml of substrate buffer. Subsequently, the strips were incubated in 2 ml of substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) for 30 min at room temperature. The aspiration of the substrate solution followed by addition of 2 ml of distilled water will stop the reaction. Following the drying step, the strips results were interpreted visually according to the provided interpretation sheets.

### 2.1.26 RHA for the Identification of beta HPV genotypes

#### 2.1.26.1 PCR amplification

All 60 extracted human genome DNA from FFPET was suitable for PCR amplification for RHA-beta (skin) HPV using primer mix (in RHA kit) as shown in Table 2.2. 10 µl of extracted DNA at 10 ηg/µl concentration was added to 40 µl of mastermix as shown in Table 2.2. 50 µl of reaction mixture for each specimen was amplified by PCR according to conditions stated in Table 2.3. Figure 2.8 showing the PM primer mix (*commercially unavailable*) target region (shaded DNA sequences) in E1 protein gene of HPV 8 as one of the candidate for beta HPV genotyping (117 bp amplimer was generated).

```
LOCUS       PPH8CG                  7654 bp    DNA     circular VRL 15-JUL-1998
DEFINITION  Human papillomavirus type 8, complete genome.
ACCESSION   M12737
VERSION     M12737.1  GI:333074
KEYWORDS    .
SOURCE      Human papillomavirus type 8
  ORGANISM  Human papillomavirus type 8
            Viruses; dsDNA viruses, no RNA stage; Papillomaviridae;
            Betapapillomavirus.

    ......
    ......

     2581 atccttttcc aatgaaacca gacaatacac ctgaatttga attaactgac caaagctgga
     2641 aatcttttt tgcaaggctt tggacacaat tagagctgag tgatcaagaa gacgagggcg
     2701 aacatggaga atctcagcga gcgtttcaat gttctgcaag atcagctaat gaacatttat
     2761 gaagctgcag aacaaacact tgaggcacag attgcgcatt ggctgctttt gcgaaaagaa

    ......
    ......
```

**Figure 2.8: The target region in E1 protein gene for beta HPV genotyping**

2.1.26.2 HPV Genotyping

A shaking water bath was heated to exactly 50°C and a calibrated thermometer was utilised to counter check the temperature. Distilled water was used and the water level adjusted to between 1/3 - 1/2 of the trough height. Hybridisation solution and Stringent Wash solution must be prewarmed at 37°C – 50°C and all crystals should be dissolved by mixing prior being used.

The test strip was removed from the tube using forceps and labelled using a pencil according to sample identification number above the line of the strip. Care should be taken not to touch below the line of the strip with bare hand to avoid any contamination. 10 µl of denaturation solution was pipetted into the upper corner of each labelled trough followed by 10 µl 3B-Buffer using sterile aerosol pipette tips. Then, 10 µl amplified biotinylated PCR product was added and the solutions were carefully mixed by pipetting. The denaturation process was allowed to proceed for 5 min at ambient temperature.

The prewarmed Hybridisation Solution was shaken and gently 2 ml was added into each trough and was mixed by gentle shaking. Care should also be taken not to contaminate the adjacent troughs during pipetting. The labelled strip was immediately placed in the labelled test trough (one strip per trough and in total eight troughs per tray). The strips should be facing up as the label at the top and completely submerged in the solution. Splashing water from the water bath into the trough should be avoided. The tray is placed in the 50°C shaking water bath and the lid was closed and incubated at 80 rpm agitation for 60 min.

The tray was removed from the water bath after incubation in hybridisation solution. The tray was held at a low angle and the mixture was aspirated from the trough using a pipette. 2 ml pre-warmed Stringent Wash solution was added and the tray was gently shaken for 10 to 20 sec at ambient temperature for rinsing. The solution was aspirated from each trough. This washing step was repeated once. Finally, each strip was incubated in 2 ml pre-warmed Stringent Wash solution in the shaking water bath at $50^{o}$C for 30 min and the lid of the water bath was closed. The tray was removed from the water bath after stringent wash incubation. The mixture was aspirated from each trough and each strip was washed twice for 1 min using 2 ml of Rinse Solution. The solution was pipetted out and 2 ml of Conjugate Solution was added to each trough and was incubated for 30 min on the shaker at ambient temperature (20 to $25^{o}$C).

The tray was removed from the shaker after the conjugate incubation, and each strip was washed twice for 1 min using 2 ml diluted Rinse Solution. It was followed with a washing step using 2 ml Substrate Buffer and the mixture was aspirated. 2 ml Substrate Solution (already kept in the dark) was added to each trough and the tray was kept in a container wrapped with foil (to maintain dark environment). The covered container was incubated for 30 min on the shaker at 80 rpm agitation at room temperature.

The container was removed from the shaker after the colour development incubation and the solution was aspirated. The developing colour was stopped by washing twice with 2 ml distilled water while gentle shaking for at least 3 min. The strips were removed from the troughs using forceps and were placed in between tissue

towel to allow them to dry completely. Each strip was stuck to the interpretation sheet by aligning the conjugate control line.

## 2.2 THE ROLE OF p16 IN ORAL DISEASE PROGRESSION

### 2.2.1 Introduction

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions. One of the biggest problems with immunohistochemistry has been how to maintain good morphology and also the immunoreactivity of antigens in paraffin embedded tissue sections. The antigen-masking effects of formaldehyde fixation are reversible to varying degrees by a process known as antigen retrieval. Antigen retrieval involves exposing the tissue sections to heat or proteolytic enzyme digestion before commencing immunochemical staining. The mechanisms of antigen retrieval are poorly understood but this method is believed to break the methylene bridge cross-links formed between proteins during formaldehyde fixation, thus allowing the proteins to take on a more tertiary-like structure and allowing antibodies access to the epitope. The schematic representation of the immunochemical staining technique after antigen retrieval is shown in Figure 2.9.

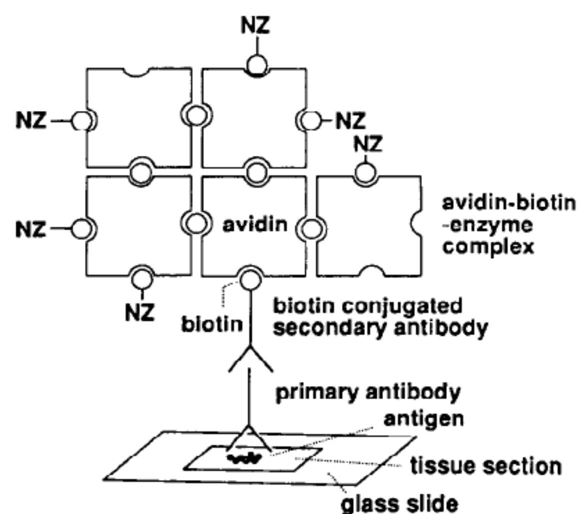



**Figure 2.9: ABC immunochemical staining procedure**
(Haines and Chelak, 1991)

Therefore, antigen retrieval by pre-treatment during optimisation is the vital stage in the technique prior to incubation with the optimal dilution of the antibody. The optimum dilution for an antibody is the highest at which specific immunoglobulin saturates the available antigen, leaving some unbound antibody in the solution to ensure continued binding.

### 2.2.2 Aim

Numerous studies have revealed that the development of oral cancer is associated with high-risk human papillomavirus (HR-HPV) types. The HPV E7 oncoprotein inactivates the pRB tumour suppressor protein and increases the expression of $p16^{INK4a}$, a cyclin-dependent kinase inhibitor, by the loss of negative feedback control.

The aim of this study was to investigate the expression of p16 in 60 FFPETs oral squamous cell carcinomas. From the pilot study results, we could correlate the positivity of p16 expression with HPV status and will be discussed in chapter 3. In addition, we increased the sample size to 176 (which consisted of benign, dysplasia and carcinoma specimens) to assist us with a better understanding of oral disease progression involving those two biomarkers and this will be further discussed in chapter 4, 5 and 6.

### 2.2.3 General Equipment

*Photographs of selected major instruments used are as shown in Figure 2.10.*

- An automated microtome, Shandon Citadel 2000, Micron HM335E
- A microwave, Tecnolec, T250t: 750 Watts

- A 3D rocking platform, Stuart Scientific STR9
- A microscope, Leica Microscopy Systems Ltd (020-519.502)
- A microscope mounted digital camera, Leica DC100
- A microscope, Motic BA400 Biological Microscope, Motic Instruments Inc. Canada
- Episcopic-Fluorescence Attachment EF-UPR-III for BA400, Motic Instruments Inc. Canada
- A computer installed with Motic Image Plus 2.0 ML (Motic China Group Co., Ltd.)

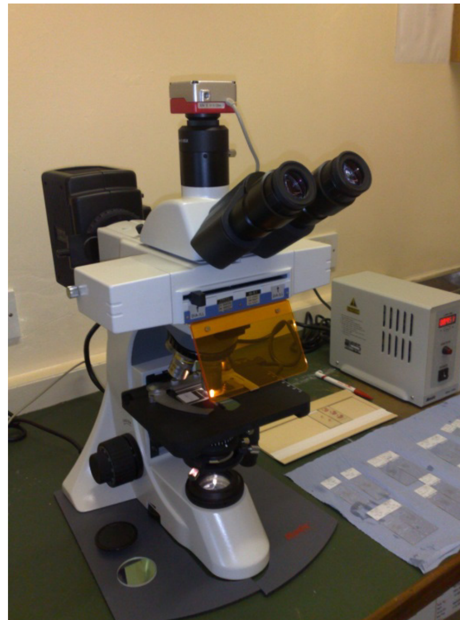


**Figure 2.10: Motic BA400 Biological Microscope**

### 2.2.4 Specimens for p16 immunohistochemistry staining

#### 2.2.4.1 Optimisation of p16 immunohistochemistry.

Initially, eight paraffin blocks were randomly selected which consisted of normal oral mucosa (n=1), kidney (n=1), skin (n=1), papilloma (n=1), oral squamous carcinoma (n=4), to optimise p16 expression. Nine paraffin blocks were randomly selected for comparing two primary antibodies. The first two were carcinoma of the oral and human salivary gland specimens. The second four samples were positively identified containing HPV DNA by PCR and HPV genotyping. The third three samples were negative for HPV DNA.

#### 2.2.4.2 Pilot study: Oral squamous cell carcinoma specimens for p16 IHC staining (*n*=60)

Sixty paraffin blocks of squamous cell carcinoma previously selected for HPV investigation were used for p16 expression based on the optimal condition achieved (Appendix 13). A negative control for p16 IHC (*n*=1) was selected for each run of 24 FFPET specimens.

#### 2.2.4.3 The role of p16 in disease progression

A hundred and seventy six paraffin blocks of mixed pathological specimens previously selected for HPV investigation were used for p16 expression based on the optimal conditions achieved. The mixed pathological specimens were (*n*=176; benign, *n*=84;

dysplasia, *n*=12; carcinoma, *n*=80). We utilised another seven normal tissue blocks for comparison of p16 expression. A negative control for p16 IHC (*n*=1) was selected for each run of 24 FFPET specimens..

### 2.2.5 Materials for immunohistochemistry staining

- Polysine$^{TM}$ glass slides, VWR International (Cat. no. 631-0107)
- Coverslip autoslip coverglass, (Cat. no. 67761323)
- ImmEdge pen, (Cat. no. H-4000), Vector Laboratories Ltd., Peterborough, UK.
- DePeX (Distrene, plasticizer, xylene/dibutyl phthalate xylene) mounting medium for microscopy, (Lot. 840316160), BDH Chemicals Ltd. , Poole, England
- Xylene, VWR Ltd, Lutterworth, Leicestershire, UK
- 95% and 100% alcohol, Chemistry Store, University of Dundee
- 30% (v/v) Hydrogen peroxide, (Cat. no. 285194H), VWR Ltd, Lutterworth, Leicestershire, UK.
- Phosphate buffered saline, pH 7.5 (PBS), (Cat. no. P4417), Sigma-Aldrich Ltd, Poole, UK.
- Citrate buffer (2.1g of anhydrous citric acid dissolved in 1 litre of distilled water) (pH=6.0 with 1M of NaOH) (Cat. no. 44445), BDH Laboratory supplies, Poole, UK.
- Retrievagen A (pH6) (Cat. no. 550524), BD Pharmingen, BD Biosciences, Inc. USA.

- Normal Goat Serum (NGS) (Cat. no. S-1000, Lot. No. T0504), Vector Laboratories Ltd., Peterborough, UK.
- Normal Mouse IgG1, Dako, X0931 (Lot. 00044506) (Negative Control)
- Protease type XXIV: Bacterial (P24), (Cat. no. P8038, Lot. 85H1192), Sigma, UK.
- Mouse Anti-Human p16 (JC8) (Cat. no. sc-56330), Primary antibody, Santa Cruz Biotechnology, Inc. (USA).
- Purified Mouse Anti-Human p16 (INK4) (Cat. no. 550834), BD Pharmingen, Primary antibody, BD Biosciences, Inc. USA.
- Mouse ABC Staining Systems (Cat. no. sc-2017), Santa Cruz Biotechnology, Inc. (USA).
- Vectastain ABC Kit, Standard Elite (PK6100), Vector Laboratories Ltd., Peterborough, UK.
- Goat Anti- MouseIgG/Biotinylated, (Cat. no. BA-9200, Lot no. T0206), Secondary antibody, Vector Laboratories Ltd., Peterborough, UK.
- Scott’s tap Water Solution (STWS) blueing agent, (Cat. no. PS138/D), Bios-Europe, Lancashire, UK.
- DAB (3,3’ diaminobenzidine) solution, (Cat. no. D5637), Sigma-Aldrich Ltd., Poole, UK.
- Mayer’s Haematoxylin, (Cat. no. PS50/C), Bios-Europe, Lancashire, UK.

### 2.2.6 The tissue specimens preparation

A microtome was used to cut 5 μm sections from FFPET blocks. Later, the multiple sections were gently placed into a water filled Electrothermal Paraffin Section Mounting Bath (already prewarmed at 55$^{o}$C) to avoid the formation of folding artifacts.

An individual section was mounted onto the Polysine slide by placing it in the water bath at an acute angle beneath the floating section and attaching it to approximately the middle of the slide by adhesion. All mounted slides were placed in a metal rack and kept in the oven at 65$^{o}$C for at least 2 hours to remove excess paraffin and to prevent the section floating off during processing. Next, all slides were labelled in pencil according to antibody, dilution, no pre-treatment, pre-treatment and date. Finally, all slides were stored into a metal or plastic slide rack for future staining steps.

### 2.2.7 p16 immunohistochemistry staining protocol

It was important to perform the pre-treatment of the paraffin sections to unmask tissue antigens as was mentioned earlier in 2.2.1. Therefore, several pre-treatments were undertaken to determine which was the most suitable for the p16 antibody: primary antibody dilution, protease XXIV (P24), microwave and autoclave. Two primary antibodies were tested throughout p16 IHC optimisation, p16 (JC8), Santa Cruz (Cat. no. sc-56330) and p16 (INK4): BD Pharmingen, (Cat. no. 550834). The overall optimisation strategy for p16 expression is illustrated in Figure 2.11.

2.2.7.1 General protocol – Day 1

- All slides were grouped according to primary antibody used at 1:10, 1:25, 1:50 and 1:100 dilutions and each with sub-groups (no pre-treatment, enzymatic, microwave and autoclave pre-treatments) (Figure 2.11).
- For optimisation purposes an initial dilution of 1:10 dilution of primary antibody was used. The optimum condition achieved through semi-quantitative assessments would assist for the selection of suitable dilution.
- The microwave pre-treatment slides were placed in water for 2 min.

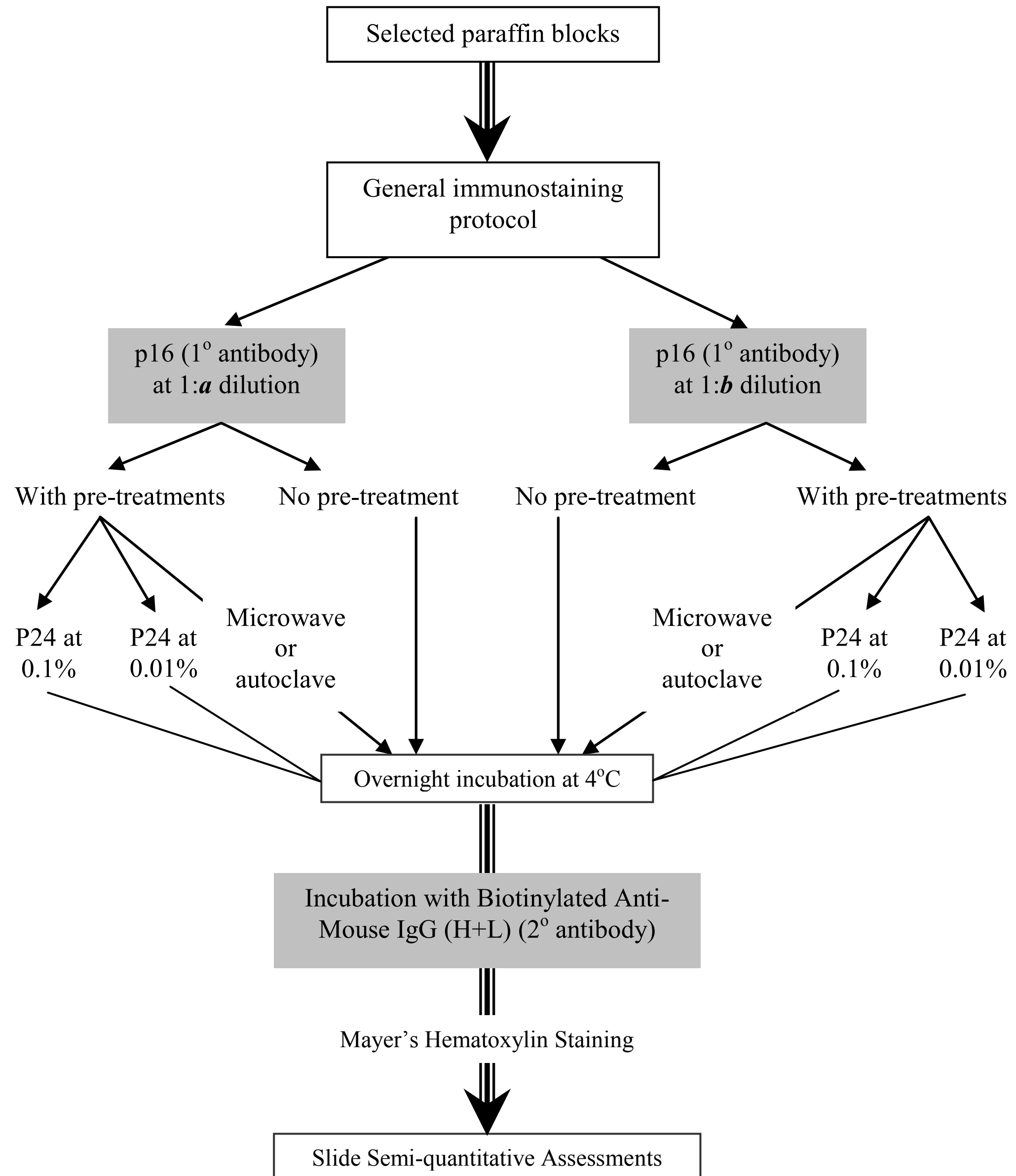



**Figure 2.11: Key optimisation steps for p16 expression**

- All slides (including microwave pre-treatment slides) were deparaffinised in xylene for 5 min.
- The slides were rehydrated in graded alcohol (absolute alcohol, 95% alcohol, and 70% alcohol) for 5 min each.
- Later, the slides were rinsed for 5 min under tap water.

- 1% (v/v) hydrogen peroxide ($H_2O_2$) in PBS was prepared [8 ml of 30% (v/v) $H_2O_2$ in 240 ml of PBS].
- The slides were placed in 1% (v/v) $H_2O_2$ in PBS for 20 min to block endogenous peroxidase activity.
- The slides were washed with two changes of 1X PBS (approximately 300 ml of 1X PBS required for each wash).
    - All washing steps in PBS were carried out with agitation on top of gyro-rocker for 5 min each.
- The section for each slide was outlined with PAP pen to prevent spillage of solutions.
- All slides with pre-treatments were undertaken to section 2.2.7.2 whereas for none pre-treated slides to section 2.2.7.3.

2.2.7.2 Pre-treatments – Day 1

a. Enzymatic treatment, Protease XXIV (P24)

- 0.1% (v/v) and 0.01% (v/v) P24 were prepared [1% (w/v) P24 stock diluted with 1X PBS].
- 100 μl of 0.1% (v/v) or 0.01% (v/v) P24 was added to the assigned slides at the staining tray.
- The slides were transferred into a staining box containing damp tissues and were incubated at 37$^{o}$C for 20 min.
- The slides were returned to the rack and washed with two changes of PBS.

b. Microwave treatment (with citric acid)

- The slides were placed in a plastic holder in a plastic box.
- The citric acid buffer was added until the slides were submerged.
- The plastic box was covered with cling film and was placed in the microwave.
- The microwave was set at full power for 5 min.
- More buffer was added if the slides were not submerged and microwaved for a further 5 min.
- The slides were allowed to cool down for 10 min and were rinsed in deionised water.
- The slides were returned to the rack and washed with two changes of PBS.

c. Autoclave treatment (with Retrievagen A, pH 6.0)

- The slides were placed in the plastic coplin jar.
- A working solution of Retrievagen A was prepared by adding 18 ml of Retrievagen A solution 1 and 82 ml of Retrievagen A solution 2 and was brought the final volume to 1 liter in distilled water.
- The working solution of Retrievagen A, pH 6.0 was added until the slides were submerged.
- The slides in the plastic coplin jar were autoclaved for 10 min.
- The coplin jar with the slides was removed and the jar was covered tightly.
- The solution was allowed to cool down for 20 min at room temperature and the slides were rinsed in three changes of PBS, 5 min each.

2.2.7.3 General protocol

Day 1 (after section 2.2.7.2 for slides with pre-treatments)

- All slides were placed in the staining tray containing damp tissues to maintain humidity.
- NGS in PBS at 1:5 dilutions was prepared and applied to each slide at room temperature for 20 min to block non-specific staining.
- Excess NGS/PBS (1:5) was removed before adding the diluted primary antibody.
- Specific dilution of primary antibody was made up with diluted NGS/PBS (1:5).
- 100 µl of diluted primary antibody was added to completely cover each section.
- The staining tray was covered with a lid to avoid evaporation.
- All slides were incubated at 4$^{o}$C in the fridge overnight (or at room temperature for 30 min).

Day 2 (next morning):

- The primary antibody was rinsed off in a stream of PBS.
- All slides were washed with two changes of PBS and finally excess PBS was removed.
- The sections were incubated with NGS/PBS (1:5) for 10 min at room temperature and excess fluid was removed.
- Biotinylated secondary antibody (6 µl of anti-mouse IgG in 1 ml NGS/PBS (1:5) dilution) was prepared.
- Each slide was incubated in 100 µl of diluted secondary antibody for 30 min at room temperature.

- Meanwhile, ABC staining system was prepared 30 min before it was required by mixing 12 µl of reagent A (Avidin) and 12 µl of reagent B (Biotin) in 1 ml of PBS and was kept at room temperature until needed.
- The slides were rinsed in a stream of PBS and were washed with two changes of PBS.
- Excess fluid was removed and 100 µl of ABC was added to completely cover each section and then incubated at ambient temperature for 30 min.
- The slides were rinsed in a stream of PBS and were washed with two changes of PBS.
- The slides were immersed in a solution made up of 1600 µl DAB, 400 µl $H_2O_2$ in 400 ml PBS.
    - DAB was completely thawed without any suspension left at the bottom of the vial (heated with water) before mixing the solution.
    - The DAB-containing solution (from staining box) was tested by mixing together with excess ABC and the solution should turn to dark brown.
    - Used DAB-containing solution was removed into the designated disposal bottle whereas tips and the vials were removed into the designated yellow bin.
- Later, the slides were washed for 2 min under tap water.
- The slides were counter-stained with Mayer's Haematoxylin for 1 min.
- The specimens were rinsed for 1 min under tap water until the water ran clear.
- The specimens were placed in STWS blueing agent for 1 min and were rinsed in running water for 1 min.
- The slides were placed and dehydrated successively in 70% alcohol, 95% alcohol and 100% alcohol for 2 min each.

- Finally, the slides were immersed in xylene for 2 min and were mounted with DePeX.

### 2.2.8 Optimisation of p16 expression

Two ABC Staining Systems were tried throughout the optimisation procedures. The first was ABC Staining Systems from Santa Cruz Biotechnology, Inc. Reagents supplied in the kits (sufficient for 200 slides) include 1.0 ml normal blocking serum, 250 mg biotinylated secondary antibody, 0.5 ml each avidin and biotinylated horseradish peroxidase (AB reagents), 1.0 ml 50x peroxidase substrate, 1.0 ml 50x DAB chromogen and 3.0 ml 10x substrate buffer. The second was Vectastain ABC Staining Systems from Vector Laboratories, Petersborough.

#### 2.2.8.1 Trial 1: Optimisation of p16 immunohistochemistry.

Eight paraffin blocks were randomly selected as mentioned in 2.2.7 to optimise p16 expression using only p16 primary antibody and mouse ABC Staining Systems from Santa Cruz.

#### 2.2.8.2 Trial 2: Comparing p16 primary antibody from two different suppliers.

Nine paraffin blocks were randomly selected as mentioned in 2.2.7. The primary antibody from a different supplier was used i.e. p16 (JC8), Santa Cruz (Cat. no. sc-56330) and p16 (INK4): BD Pharmingen, (Cat. no. 550834). Vectastain ABC Staining Systems from Vector Laboratories, Petersborough was utilised in this section.

### 2.2.9 Pilot study (*n*=60)

Sixty paraffin blocks, similar FFPETs used for HPVs study as mentioned in 2.1.4. Selected primary antibody, best ABC Staining System and optimum pre-treatment from 2.2.8 was utilised in this section.

### 2.2.10 Oral disease progression study (*n*=176)

A hundred and seventy six paraffin blocks, similar FFPETs used for HPVs study as mentioned in 2.1.4. Similarly, selected primary antibody, best ABC Staining System and optimum pre-treatment from 2.2.8 was utilised in this section.

### 2.2.11 Semi-quantitative scoring for p16 immunohistochemistry staining

A microscope (Motic BA400) with an eyepiece grid (Mertz) containing 100 points (the total size of the grid surface area is 0.476 $mm^2$) was used to assess the sections. Scanning the sections at low power (X100, 10x eyepiece and 10x objective lens power) to identify the area of positive p16 stained to identify the 'hot spot' across the length of the epithelium without overlapping. The mean of randomly selected 10 'hot spot' fields were assessed at X400 magnification (10x eyepiece and 40x objective lens power) for intensity and percentage of staining taken as an estimate (10% cut-off point for p16 positivity) (Angiero et al., 2008; Tanaka et al., 2005). Strong nuclear and cytoplasmic staining defined a positive reaction. The p16 positive staining was limited to the tumour cells, occasionally including a few multinucleate giant cells which stained strongly positive, and less than 5% epithelium had focal positive stain. The p16 positive

distribution was scored on a semi-quantitative scale at first and then converted to positive and negative scores before statistical analyses. All p16 slides were examined and scored by two observers and 10% were re-reviewed by a third observer. A positive reaction by non-tumour cells, such as multinucleated giants cells and epithelia, were not included as positive.

### 2.3 STATISTICAL ANALYSIS

Data was entered into an Excel 2007 spreadsheet (Microsoft Corp., USA) and imported into a software package, Statistical Package for Social Sciences (SPSS, version 16, Inc. Chicago, Illinois, USA). The Kolmogorov-Smirnov *Z* statistic with Lilliefors significance level was performed to test normality of the data. If the significance level is greater than 0.05, then the normality is assumed. The Shapiro-Wilks statistic is also calculated if the sample size is less than fifty. The data was later analysed depending on the distribution, parametric analysis if normally distributed or non-parametric analysis if not normally distributed. Wilcoxon matched-pairs test for two related parameters and Friedman's repeated measures two-way analysis of variance (ANOVA) for multiple related parameters was utilised to examine the variation which exist inter-observer. A Spearman's Rank Order Correlation (rho) test was used to correlate the strength of the relationship between two variables. Comparisons between tissues were carried out using Mann-Whitney *U* test with a 95% confidence interval. Kaplan-Meier (product estimates) and log rank tests were used for survival analysis for each of the variables and clinical factors. Cox regression analysis was performed to identify whether any of these factors were of independent prognostic significance if $p$ value was less than 0.05 for the differences.

# CHAPTER 3

# RESULTS OF PILOT STUDIES

## 3.1 OVERVIEWS

Overviews of aims of this chapter are two fold. The **first** is to optimise the methodology specifically to answer the following questions:

- The quality and quantity of the extracted DNA using positive controls
- The quality and quantity of the extracted DNA from FFPETs
- The effect of DNA volume on the DNA extraction
- A comparison of DNA extraction methods (Qiagen versus Promega kit)
- Optimisation of Polymerase Chain Reaction
  - β-globin detection by PCR for DNA quality determination
  - PCR optimisation for L1 HPV detection

The **second** and major aim of this pilot study was:

- To extract DNA from 60 cancers (FFPETs)
- PCR amplification for L1 HPV detection
- Purification and DNA sequencing of the PCR products
- Alpha and beta (β-HPV) Genotyping
- HPV prevalence and clinicopathological parameters
- Correlation among demographic and clinical parameters associated with HPVs

The **third** aim is to investigate:

- The potential role of p16 in the same 60 cancers as above

- Possible correlations among demographic and clinical parameters associated with p16
- Correlation between HPVs and p16 expression
- Potential association with survival for HPV and p16

## 3.2 OPTIMISATION OF DNA EXTRACTION

### 3.2.1 The quality and quantity of the extracted DNA of positive controls

#### 3.2.1.1 Repeated measurements within positive controls (HSGs)

The raw data for qualitative and quantitative DNA extracted from human salivary glands (A1 and A2) was given in Appendix 6, Table A. Mann-Whitney U and Wilcoxon W tests were performed to investigate the variation which could exist from the duplication of the same sample. Kruskal Wallis test on the other hand, was performed for repeated measurements effect. The result of statistical analysis is shown in Table 3.1a and 3.1b.

**Table 3.1a: The effect of duplication**

**Test Statistics[b]**

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Mann-Whitney U | 11.000 | 9.000 | 14.000 | 14.500 |
| Wilcoxon W | 32.000 | 30.000 | 35.000 | 35.500 |
| Z | -1.131 | -1.444 | -0.642 | -0.562 |
| Asymp. Sig. (2-tailed) | 0.258 | 0.149 | 0.521 | 0.574 |
| Exact Sig. [2*(1-tailed Sig.)] | 0.310[a] | 0.180[a] | 0.589[a] | 0.589[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: HSG (A1 & A2)

**Table 3.1b: The effect of replication**

**Test Statistics[a,b]**

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Chi-Square | 0.480 | 0.203 | 1.341 | 0.358 |
| *df* | 2 | 2 | 2 | 2 |
| Asymp. Sig. | 0.787 | 0.904 | 0.511 | 0.836 |

*a*. Kruskal Wallis Test

*b*. Grouping Variable: Replication

3.2.1.2 Comparison between two positive controls (cells)

The raw data for qualitative and quantitative DNA extracted from human salivary glands (A1 and A2) and 3T3 embryonic mouse fibroblasts (B1 and B2) was given in Appendix 6, Table B. Mann-Whitney U, Wilcoxon W and Kruskal Wallis tests were performed as in section 3.2.1.1 to investigate the variation which could exist from similar sample type (cells). The result of statistical analysis is shown in Table 3.2a and 3.2b.

**Table 3.2a: Comparison between two positive controls**

**Test Statistics[b]**

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Mann-Whitney U | 10.500 | 16.000 | 17.000 | 9.000 |
| Wilcoxon W | 31.500 | 37.000 | 38.000 | 30.000 |
| Z | -1.205 | -0.323 | -0.160 | -1.444 |
| Asymp. Sig. (2-tailed) | 0.228 | 0.747 | 0.873 | 0.149 |
| Exact Sig. [2*(1-tailed Sig.)] | 0.240[a] | 0.818[a] | 0.937[a] | 0.180[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: HSG & 3T3

**Table 3.2b: The effect of replication**

**Test Statistics**[a,b]

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Chi-Square | 0.203 | 0.468 | 0.731 | 0.261 |
| *df* | 2 | 2 | 2 | 2 |
| Asymp. Sig. | 0.903 | 0.791 | 0.694 | 0.878 |

*a*. Kruskal Wallis Test

*b*. Grouping Variable: Replication

#### 3.2.1.3 Preliminary conclusions

From 3.2.1.1, all parameters assessed in the duplication of the same sample and the repeated measurements taken for quality and quantity of the extracted DNA were not statistically significant different ($p$>0.05). Similarly, it was demonstrated in 3.2.1.2 that the variation in similar type of sample (cells) and repeated readings were not statistically significant. Therefore, it was sufficient to take only a single measurement for each sample for DNA quality and quantity comparison purposes.

### 3.2.2 The quality and quantity of the extracted DNA from FFPETs

#### 3.2.2.1 Repeated measurements within FFPETs

The raw data for qualitative and quantitative DNA extracted from FFPETs (C1 and C2) was given in Appendix 7, Table A. Mann-Whitney U and Wilcoxon W tests were performed to investigate the variation which could exist from the duplication of the same sample. Kruskal Wallis test on the other hand, was performed for repeated measurements effect. The result of statistical analysis is shown in Table 3.3a and 3.3b.

**Table 3.3a: The effect of duplication**

**Test Statistics**[b]

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Mann-Whitney U | 18.000 | 18.000 | 3.000 | 18.000 |
| Wilcoxon W | 39.000 | 39.000 | 24.000 | 39.000 |
| Z | 0.000 | 0.000 | -2.406 | 0.000 |
| Asymp. Sig. (2-tailed) | 1.000 | 1.000 | 0.016 | 1.000 |
| Exact Sig. [2*(1-tailed Sig.)] | 1.000[a] | 1.000[a] | 0.015[a] | 1.000[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: FFPETs (C1 & C2)

**Table 3.3b: The effect of replication**

**Test Statistics**[a,b]

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Chi-Square | 0.155 | 0.155 | 1.245 | 0.010 |
| *df* | 2 | 2 | 2 | 2 |
| Asymp. Sig. | 0.925 | 0.925 | 0.537 | 0.995 |

*a*. Kruskal Wallis Test

*b*. Grouping Variable: Replication

#### 3.2.2.2 Comparison amongst FFPETs

The raw data for qualifying and quantifying DNA extracted from randomly selected FFPETs (C1 to C6) was given in Appendix 7, Table B. Kruskal Wallis test was performed similar to section 3.2.1.1 to investigate the variation which could exist from similar sample type (FFPETs). The result of statistical analysis is shown in Table 3.4a and 3.4b.

**Table 3.4a: Comparison amongst randomly selected six FFPETs**

**Test Statistics**[a,b]

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Chi-Square | 16.103 | 15.862 | 8.251 | 15.971 |
| *df* | 5 | 5 | 5 | 5 |
| Asymp. Sig. | 0.007 | 0.007 | 0.143 | 0.007 |

*a*. Kruskal Wallis Test

*b*. Grouping Variable: FFPETs (C1-C6)

**Table 3.4b: The effect of replication**

**Test Statistics**[a,b]

| | $A_{260}$ | $A_{280}$ | DNA purity | DNA yield |
|---|---|---|---|---|
| Chi-Square | 0.038 | 0.073 | 0.667 | 0.152 |
| *df* | 2 | 2 | 2 | 2 |
| Asymp. Sig. | 0.981 | 0.964 | 0.717 | 0.927 |

*a*. Kruskal Wallis Test

*b*. Grouping Variable: Replication

#### 3.2.2.3 Preliminary conclusions

From 3.2.2.1, the difference was not statistically significant in all parameters in the duplication of the same sample and the repeated measurements taken for quality and quantity of the extracted DNA except for DNA purity ($p<0.05$). Similarly, it was demonstrated in 3.2.2.2 that repeated readings were not statistically significantly different. In contrast, the variation of DNA yield in similar type of sample (FFPETs) were statistically significant ($p<0.01$). Therefore, from now onwards we would consider an average of the measurements for each sample for DNA quality and quantity comparison purposes.

### 3.2.3 The effect of DNA volume to the DNA extraction

#### 3.2.3.1 DNA volume effect using Qiagen kit

The raw data for qualifying and quantifying DNA extracted from three different volumes (3, 5 and 10 coupes) using Qiagen kit was given in Appendix 8 and 9. One-Way ANOVA analysis was performed and later with Post Hoc Tests if the overall $F$ significant. If the Levene test (Test of Homogeneity of Variance) was significant, Games-Howell test was performed or the Tukey HSD (honestly significant differences) test, if Levene test was not significant. It was revealed that DNA quantity was not significantly different in the three DNA volumes. Meanwhile, DNA purity of 10 coupes was significant higher ($p<0.05$) than 3 coupes. In addition, DNA purity of either 5 coupes to 3 coupes or 5 coupes to 10 coupes was comparable statistically.

#### 3.2.3.2 DNA volume effect using Promega kit

The raw data for qualitative and quantitative DNA extracted from three different volumes (3, 5 and 10 coupes) using Promega kit was given in Appendix 10 and 11. One-Way ANOVA analysis was performed and will proceed with appropriate Post Hoc Tests. The result of statistical analysis demonstrated that the DNA quality and quantity were statistically comparable amongst the three DNA volumes using Promega kit. Since overall $F$ was not significant, Post Hoc tests was not required.

#### 3.2.3.3 Preliminary conclusions

From 3.2.3.1 and 3.2.3.2, it can be concluded that DNA yield was comparable amongst three different DNA volumes using both kits separately. Similarly, DNA purity seems not significantly different in the three DNA volumes utilising Promega kit. Nevertheless, by using the Qiagen kit, 10 coupes demonstrated significantly better DNA purity than 3 coupes. Certainly, we could utilise the Qiagen kit and extracting 10 coupes to meet the highest DNA quality and quantity. Up to this point, no conclusion could be made regarding which DNA extraction kit was better for DNA purity and DNA yield for the very minimum DNA volume used.

### 3.2.4 DNA extraction methods comparison (Qiagen kit versus Promega kit)

An average of DNA purity and DNA yield in each DNA volume using two DNA extraction methods, Qiagen kit and Promega kit are summarised in Appendix 12. The Mann-Whitney U, Wilcoxon W and Two-sample Kolmogorov-Smirnov Z Tests were carried out to compare both DNA extraction kits in separate DNA volumes.

#### 3.2.4.1 Comparison using extracted DNA from 3 coupes of FFPET

Table 3.5 shows the result of statistical analysis comparison between extraction kits using a DNA volume of 3 coupes. The Promega kit was significantly better than the Qiagen kit for DNA purity. In contrast for DNA yield, Qiagen kit performed significantly better than Promega kit.

**Table 3.5: Mean comparison between two kits (3 coupes)**

(a)

**Mann-Whitney Test U: Ranks**

| | DNA extraction kits | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| DNA purity | Promega kit | 15 | 19.77 | 296.50 |
| | Qiagen kit | 15 | 11.23 | 168.50 |
| | Total | 30 | | |
| DNA yield | Promega kit | 15 | 10.10 | 151.50 |
| | Qiagen kit | 15 | 20.90 | 313.50 |
| | Total | 30 | | |

(b)

**Test Statistics[b]**

| | DNA purity | DNA yield |
|---|---|---|
| Mann-Whitney U | 48.500 | 31.500 |
| Wilcoxon W | 168.500 | 151.500 |
| Z | -2.658 | -3.361 |
| Asymp. Sig. (2-tailed) | 0.008 | 0.001 |
| Exact Sig. [2*(1-tailed Sig.)] | 0.007[a] | 0.000[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: DNA extraction kits

(c)

**Two-sample Kolmogorov-Smirnov Z: Test Statistics[a]**

| | | DNA purity | DNA yield |
|---|---|---|---|
| Most Extreme Differences | Absolute | 0.533 | 0.667 |
| | Positive | 0.000 | 0.667 |
| | Negative | -0.533 | 0.000 |
| | Kolmogorov-Smirnov Z | 1.461 | 1.826 |
| | Asymp. Sig. (2-tailed) | 0.028 | 0.003 |

*a*. Grouping Variable: DNA extraction kits

#### 3.2.4.2 Comparison using extracted DNA from 5 coupes of FFPET

Table 3.6 shows the result of statistical analysis comparison between extraction kits using a DNA volume of 5 coupes. Promega kit and Qiagen kit were comparable for DNA purity. Qiagen kit demonstrated significantly higher yields than Promega kit.

**Table 3.6: Mean comparison between two kits (5 coupes)**

(a)

**Mann-Whitney Test: Ranks**

| | DNA extraction kits | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| DNA purity | Promega kit | 15 | 13.43 | 201.50 |
| | Qiagen kit | 15 | 17.57 | 263.50 |
| | Total | 30 | | |
| DNA yield | Promega kit | 15 | 10.23 | 153.50 |
| | Qiagen kit | 15 | 20.77 | 311.50 |
| | Total | 30 | | |

(b)

**Test Statistics[b]**

| | DNA purity | DNA yield |
|---|---|---|
| Mann-Whitney U | 81.500 | 33.500 |
| Wilcoxon W | 201.500 | 153.500 |
| Z | -1.287 | -3.278 |
| Asymp. Sig. (2-tailed) | 0.198 | 0.001 |
| Exact Sig. [2*(1-tailed Sig.)] | 0.202[a] | 0.001[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: DNA extraction kits

(c)

**Two-sample Kolmogorov-Smirnov Z: Test Statistics[a]**

| | | DNA purity | DNA yield |
|---|---|---|---|
| Most Extreme Differences | Absolute | 0.267 | 0.600 |
| | Positive | 0.267 | 0.600 |
| | Negative | 0.000 | 0.000 |
| | Kolmogorov-Smirnov Z | 0.730 | 1.643 |
| | Asymp. Sig. (2-tailed) | 0.660 | 0.009 |

*a*. Grouping Variable: DNA extraction kits

#### 3.2.4.3 Comparison using extracted DNA from 10 coupes of FFPET

Table 3.7 shows the result of statistical analysis comparison between extraction kits using DNA volume of 10 coupes. Promega kit and Qiagen kit was comparable for DNA purity. Qiagen kit showed a significant higher DNA yield in comparison to Promega kit.

**Table 3.7: Mean comparison between two kits (10 coupes)**

(a)

**Mann-Whitney U Test: Ranks**

| | DNA extraction kits | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| DNA purity | Promega kit | 15 | 12.60 | 189.00 |
| | Qiagen kit | 15 | 18.40 | 276.00 |
| | Total | 30 | | |
| DNA yield | Promega kit | 15 | 10.50 | 157.50 |
| | Qiagen kit | 15 | 20.50 | 307.50 |
| | Total | 30 | | |

(b)

**Test Statistics[b]**

| | DNA purity | DNA yield |
|---|---|---|
| Mann-Whitney U | 69.000 | 37.500 |
| Wilcoxon W | 189.000 | 157.500 |
| Z | -1.808 | -3.112 |
| Asymp. Sig. (2-tailed) | 0.071 | 0.002 |
| Exact Sig. [2*(1-tailed Sig.)] | 0.074[a] | 0.001[a] |

*a*. Not corrected for ties.

*b*. Grouping Variable: DNA extraction kits

(c)

**Two-sample Kolmogorov-Smirnov Z: Test Statistics[a]**

| | | DNA purity | DNA yield |
|---|---|---|---|
| Most Extreme Differences | Absolute | 0.267 | 0.533 |
| | Positive | 0.267 | 0.533 |
| | Negative | 0.000 | 0.000 |
| | Kolmogorov-Smirnov Z | 0.730 | 1.461 |
| | Asymp. Sig. (2-tailed) | 0.660 | 0.028 |

*a*. Grouping Variable: DNA extraction kits

#### 3.2.4.4 Preliminary conclusions

The summary of the comparison between extraction kits is shown in Table 3.8. It was demonstrated that Qiagen kit performed significantly better than Promega kit for DNA yield at all DNA volumes. From the three independent test results above, we concluded that the Qiagen kit should be selected for future DNA extraction and 5 coupes of 5 μm paraffin embedded tissues is the best option for the DNA purity and DNA yield.

**Table 3.8: Summary of the comparison between extraction kits**

| DNA volume | DNA extraction kit | Mann-Whitney U Test (sig.) | | Kolmogorov-Smirnov Z Test (sig.) | |
|---|---|---|---|---|---|
| | | DNA purity | DNA yield | DNA purity | DNA yield |
| 3 coupes | Promega<br>Qiagen | 0.007** | 0.000** | 0.028* | 0.003** |
| 5 coupes | Promega<br>Qiagen | 0.202 | 0.001** | 0.660 | 0.009** |
| 10 coupes | Promega<br>Qiagen | 0.074 | 0.001** | 0.660 | 0.028* |

Note: ** - the mean different is significant at the 0.01 level, * - the mean different is significant at the 0.05 level

## 3.3 OPTIMISATION OF POLYMERASE CHAIN REACTION

### 3.3.1 β-globin detection by PCR for DNA quality determination

PCR amplifications were carried out in triplicate for each diluted DNA from 2.1.21 according to the procedure mentioned in 2.1.22, Materials and Methods. Figure 3.1 shows the sample of 2% agarose gel electrophoresis of 268 bp amplicons.

**Figure 3.1: β-globin detection in 2% agarose gel electrophoresis.** Well 1–10 = selected 268 bp amplicons, M (100 bp DNA ladder marker).

### 3.3.2 PCR optimisation for L1 HPV detection

#### 3.3.2.1 First round Semi-nested/Nested PCR – MY11/MY09 primer pair (450 bp amplicons)

<u>DNA template concentration effect</u>

Multiple serial dilution of the initial known DNA template concentration (from 0.01 ng to 100 ng) of the positive control (HPV6 DNA) was performed in triplicate to investigate the DNA yield effect. It was observed that 0.01 ng of DNA was the minimum quantity detected in a 2% agarose gel (Figure 3.2).

450 bp

**Figure 3.2: The effect of DNA template concentration in 2% agarose gel.** Well 1 = Negative control (RNAse Free water as a template DNA); Well 2–4 = 0.01 ng; Well 5–7 = 0.1 ng; Well 8–10 = 1 ng; Well 11–13 = 10 ng; Well 14-16 = 50 ng; Well 17-19 = 100 ng. M = 100 bp DNA ladder marker.

#### 3.3.2.2 Second round Semi-nested PCR – MY11/GP6 primer pair (190 bp amplicons)

<u>$MgCl_2$ concentration effect</u>

The effect of $MgCl_2$ concentration using undiluted 450 bp amplicons was tested in triplicate (Figure 3.3). It was found that at 2.5 mM $MgCl_2$ concentration (commercial premix) produced relatively more specific amplicons than 3.0 mM $MgCl_2$ (minimum

concentration). This suggested that 2.5 mM $MgCl_2$ (commercial premix) should be used, even though less bands intensity obtained for future optimisation steps.

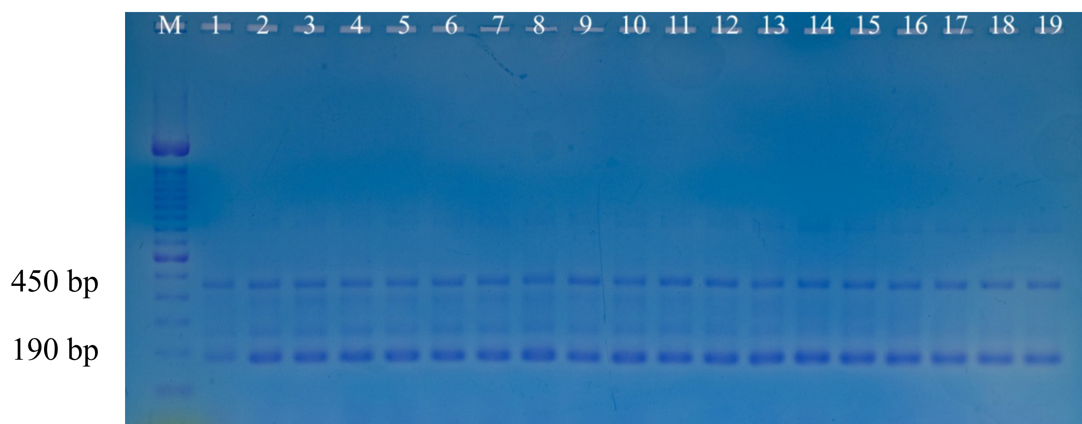



**Figure 3.3: $MgCl_2$ effect is visualised in 2% agarose gel.** Well 1 = 2.5 mM $MgCl_2$ (commercial premix); Well 2–4 = 1.5 mM $MgCl_2$; Well 5–7 = 2.0 mM $MgCl_2$; Well 8–10 = 2.5 mM $MgCl_2$; Well 11–13 = 3.0 mM $MgCl_2$; Well 14-16 = 3.5 mM $MgCl_2$; Well 17-19 = 4.0 mM $MgCl_2$. M = 100 bp DNA ladder marker.

Dilution effect of the PCR products from the first round amplification (450 bp)

Subsequently, the effect of DNA template concentration was tested at constant 2.5 mM $MgCl_2$ concentration (commercial premix) in triplicate (Figure 3.4). It was found that at $3^4$ dilution (or 81x dilution) of the 450 bp amplicon was the minimum requirement to obtain more specific 190 bp amplicons in the second round of PCR amplification. But at $3^5$ dilution (or 243x dilution), it was noted that the reaction was completely free from non-specific PCR amplicons. Therefore, it was suggested that relative comparison of the band intensity (especially 190 bp) between samples tested to the control to determine the suitable dilution of the 450 bp amplicons could be made for the next round of PCR amplification.

**Figure 3.4: The effect of DNA template concentration in 2% agarose gel.** Well 1-3 = undiluted 450 bp amplicons; Well 4–6 = $3^1$ dilution; Well 7–9 = $3^2$ dilution; Well 10–12 = $3^3$ dilution; Well 13–15 = $3^4$ dilution; Well 16-18 = $3^5$ dilution; Well 19 = 450 bp amplicon and M = 100 bp DNA ladder marker.

Figures 3.5 and 3.6 showed the effect of undiluted and diluted of selected 450 bp amplicons (14 different samples). By comparing relative band intensity amongst sample to control, approximate dilutions were made and producing specific 190 bp amplicon (Figure 3.9).

**Figure 3.5: The effect of undiluted 450 bp amplicons (as the template for second round of semi-nested PCR) in 2% agarose gel.** Well 1-14 = sample DNAs; Well 15 = positive control; and M = 100 bp DNA ladder marker.

**Figure 3.6: Effect of diluted 450 bp amplicons (as the template for second round of semi-nested PCR) in 2% agarose gel.** Well 1-14 = sample DNAs (similar sample sequence as Figure 8); Well 15 = positive control; and M = 100 bp DNA ladder marker.

3.3.2.3 Second round Nested PCR – GP5+/GP6+ primer pair (140 bp amplicons)

Dilution effect of the PCR products from the first round amplification (450 bp)

An amplicon dilution approach was used as in section 1.6.2 for nested PCR (Figure 3.7).

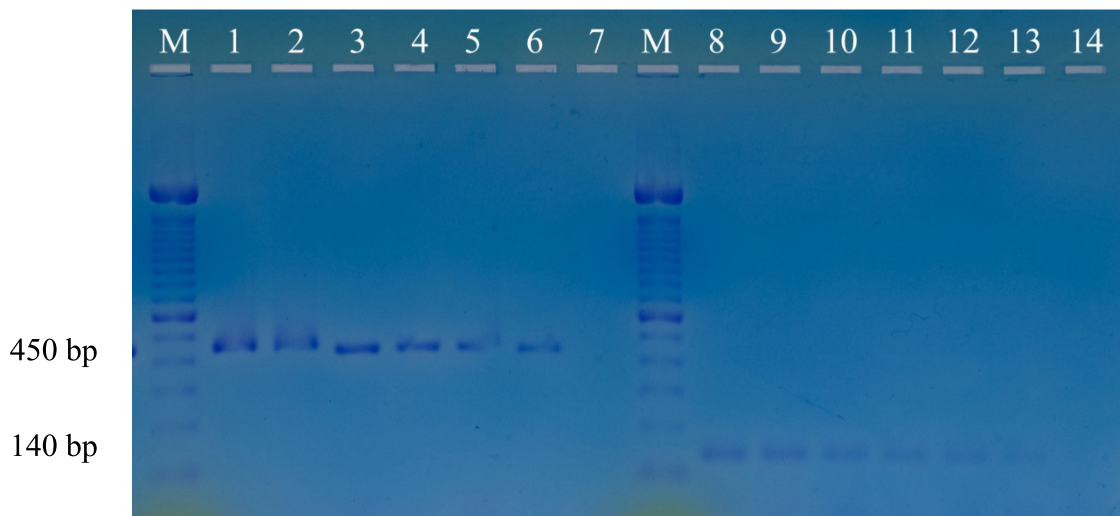



**Figure 3.7: 2% agarose gel electrophoresis of diluted 450 bp amplicons as the template for the second round of nested PCR.** Well 1–6 = Undiluted 450 bp amplicons; Well 8-13 = Nested PCR using diluted 450 bp from 1-6; Well 7 and 14 = Negative control; and M = 100 bp DNA ladder marker.

3.3.2.4 Independent PCR reaction using GP5+/GP6+ primer pair

PCR reamplification effect without additional PCR reagents

Figure 3.8 shows the effect of PCR reamplification of two selected samples in triplicate. It was found that bands intensity relatively doubled after reamplification and would be suggested for future amplification. Even though, the actual period of PCR amplification would be twice as long fortunately the 140 bp amplicons specificity was not affected.

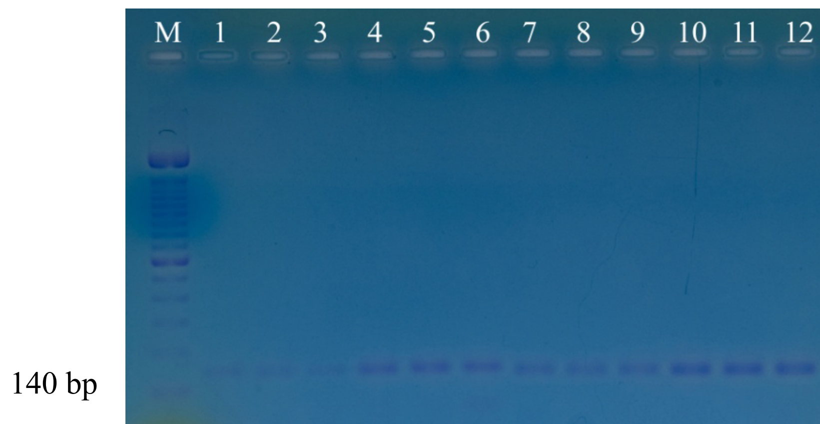



**Figure 3.8: PCR reamplification effects of 140 bp amplicons without removing samples from thermocycler.** Well 1-3 = sample 1; Well 4-6 = reamplified of sample 1; Well 7-9 = sample 2; Well 10-12 = reamplified of sample 2; and M = 100 bp DNA ladder marker.

DNA template concentration effect

The effect of DNA template concentration was determined in triplicate (Figure 3.9). It was found that 140 bp amplicons could still be detected and visualised on 2% agarose gel electrophoresis when as little as $10^{-4}$ ng of initial DNA template were used in the PCR.

140 bp

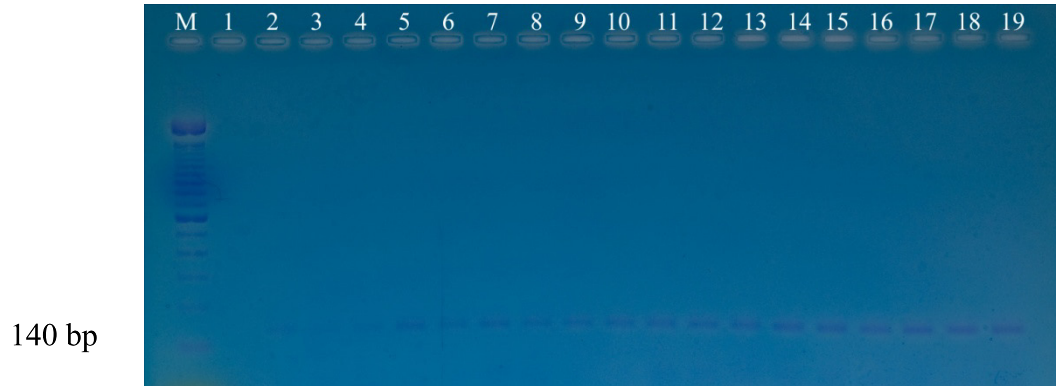



**Figure 3.9: The effect of DNA template concentration in 2% agarose gel.** Well 1 = Negative control (RNAse Free water); Well 2-4 = $10^{-4}$ ng; Well 5-7 = $10^{-3}$ ng; Well 8-10 = $10^{-2}$ ng; Well 11-13 = $10^{-1}$ ng; Well 14-16 = 1 ng; Well 17-19 = 10 ng; and M = 100 bp DNA ladder marker.

## 3.4. HPVs ROLE IN ORAL CANCER

### 3.4.1 Clinical Data

A cohort of 60 patients were studied, 56 patients with resected squamous cell carcinoma, 2 patients with carcinoma in situ and one patient each with severe dysplasia/carcinoma in situ and high grade squamous dysplasia. The male:female ratio was 33:27 with the mean age of 66.2 years, ranging from 36-97 years. The average age for male was 65.2 and 67.4 for female. Thirty five patients (58.3%) were smokers and drinkers as well, fifteen patients (25%) were non-smokers but drinkers, five patients for each group (8.3%) were smokers but non-drinkers and non-smokers and non drinkers (Table 3.9). As to the tumour sites, 29 cases were on the tongue; floor of the mouth, 10 cases; retromolar pad, 9 cases; alveolus, 4 cases; soft palate, 3 cases; and one case each for tonsil, lower lip, pharynx, supraglottis and buccal mucosa. The clinicopathological parameters recorded in each tumour consist of the primary tumour (T), regional lymph node (N), distant metastases and the stage of tumour as shown in Table 3.10.

**Table 3.9: Clinical details of oral cancer patients**

| Parameters | *N* | % |
|---|---|---|
| Total | 60 | |
| Gender | | |
| Female | 27 | 45.0 |
| Male | 33 | 55.0 |
| Age group$^{\alpha}$ (range, 36-97y) | | |
| 36-65y | 31 | 51.7 |
| 66-97y | 29 | 48.3 |
| Smoking | | |
| No | 20 | 33.3 |
| Yes | 40 | 66.7 |
| Alcohol | | |
| No | 10 | 16.7 |
| Yes | 50 | 83.3 |
| Smoking-Alcohol | | |
| No smoking-No drinking (NSND) | 5 | 8.3 |
| Smoking-No drinking (SND) | 5 | 8.3 |
| No smoking-Drinking (NSD) | 15 | 25.0 |
| Smoking-Drinking (SD) | 35 | 58.3 |
| Anatomic site | | |
| Buccal mucosa | 1 | 1.7 |
| Floor of the mouth | 10 | 16.7 |
| Alveolus | 4 | 6.7 |
| Soft palate | 3 | 5.0 |
| Pharynx | 1 | 1.7 |
| Lower lip | 1 | 1.7 |
| Retromolar pad | 9 | 15.0 |
| Supraglottis | 1 | 1.7 |
| Tongue | 29 | 48.3 |
| Tonsil | 1 | 1.7 |
| Disease status | | |
| No recurrence | 56 | 90.0 |
| Recurrence | 6 | 10.0 |
| Disease outcome | | |
| Alive | 37 | 61.7 |
| Dead | 23 | 38.3 |

*Abbreviation: N, number of cases ;$^{\alpha}$ - Age group was based on median age*

**Table 3.10: Clinicopathologic parameters of oral cancer patients**

| Parameters | *N* | % |
|---|---|---|
| Total | 60 | |
| Tumour size | | |
| Negative | 2 | 3.3 |
| $T_1$ (<2 cm) | 24 | 40.0 |
| $T_2$ (2-4 cm) | 19 | 31.7 |
| $T_3$ (>4 cm) | 1 | 1.7 |
| $T_4$ (>4 cm) and invades adjacent structures | 14 | 23.3 |
| Regional lymph nodes | | |
| Negative | 4 | 6.7 |
| $N_0$ | 28 | 46.7 |
| $N_1$ | 10 | 16.7 |
| $N_2$ | 18 | 30.0 |
| Distant metastasis | | |
| Negative | 4 | 6.7 |
| No distant metastasis | 56 | 93.3 |
| Tumour stage | | |
| 0 | 6 | 10.0 |
| I | 17 | 28.3 |
| II | 4 | 6.7 |
| III | 7 | 11.7 |
| IV | 26 | 43.3 |

*Abbreviation: N, number of cases*

### 3.4.2 DNA extraction of 60 FFPETs

The DNA extraction of 60 FFPETs was carried out using Qiagen kit in duplicate. The quantification results of the extracted DNA are shown in Appendix 13 (the duplicate was treated as a backup if the actual sample failed). The quality and quantity of the extracted DNA in each parameter is shown in Table 3.11. Each extracted DNA was further diluted accordingly with sterile distilled water ($dH_2O$) to a final concentration of 10 ng/µl for further down-stream analyses such as PCR amplification, DNA sequencing and HPV genotyping. The *t*-Test and ANOVA (Table 3.12), Mann-Whitney and Kruskal-Wallis (Table 3.13) tests were performed for means comparison. The statistical analysis revealed that DNA purity and DNA yield were not significantly different in all of the parameters compared including clinicopathological characteristics except for DNA purity of the tumour size. $T_3$ was excluded in the analysis due to the fact only a single case existed. In post-hoc tests with Tukey HSD, we found that $T_4$ was significantly lower in DNA purity compared to the negative ($p$<0.05).

**Table 3.11: The purity and yield of the extracted DNA based on parameters**

| Parameters | *N* | Mean ± $SD^{\alpha}$ | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (ng/µl) |
| Total | 60 | | |
| Gender | | | |
| Female | 27 | 1.250 ± 0.504 $^{\gamma}$ | 0.442 ± 0.226 $^{\gamma}$ |
| Male | 33 | 1.228 ± 0.476 | 0.490 ± 0.235 $^{\gamma}$ |
| Age group $^{\beta}$ | | | |
| 36-65y | 31 | 1.149 ± 0.336 $^{\gamma}$ | 0.470 ± 0.244 $^{\gamma}$ |
| 66-97y | 29 | 1.334 ± 0.597 | 0.467 ± 0.218 |
| Anatomic site | | | |
| Buccal mucosa | 1 | 0.493 | 0.147 |
| Floor of the mouth | 10 | 1.323 ± 0.372 $^{\gamma}$ | 0.445 ± 0.237 |
| Alveolus | 4 | 0.983 ± 0.442 $^{\gamma}$ | 0.372 ± 0.235 |
| Soft palate | 3 | 1.817 ± 0.991 | 0.238 ± 0.143 $^{\gamma}$ |
| Pharynx | 1 | 1.246 | 0.510 |
| Lower lip | 1 | 0.677 | 0.520 |
| Retromolar pad | 9 | 1.244 ± 0.714 | 0.552 ± 0.185 $^{\gamma}$ |
| Supraglottis | 1 | 1.056 | 0.630 |
| Tongue | 29 | 1.226 ± 0.363 $^{\gamma}$ | 0.495 ± 0.246 |
| Tonsil | 1 | 1.466 | 0.347 |
| Disease outcome | | | |
| Alive | 36 | 1.174 ± 0.326 | 0.467 ± 0.229 $^{\gamma}$ |
| Dead | 24 | 1.334 ± 0.652 | 0.472 ± 0.236 $^{\gamma}$ |
| Tumour size | | | |
| Negative | 2 | 2.005 ± 1.352 | 0.180 ± 0.061 |
| $T_1$ (<2 cm) | 24 | 1.167 ± 0.313 | 0.514 ± 0.232 $^{\gamma}$ |
| $T_2$ (2-4 cm) | 19 | 1.389 ± 0.530 | 0.472 ± 0.234 $^{\gamma}$ |
| $T_3$ (>4 cm) | 1 | 1.466 | 0.347 |
| $T_4$ (>4 cm) and invades adjacent structures | 14 | 1.029 ± 0.425 $^{\gamma}$ | 0.437 ± 0.225 $^{\gamma}$ |
| Regional lymph nodes | | | |
| Negative | 4 | 1.531 ± 0.957 | 0.423 ± 0.321 $^{\gamma}$ |
| $N_0$ | 28 | 1.204 ± 0.523 | 0.478 ± 0.233 $^{\gamma}$ |
| $N_1$ | 10 | 1.234 ± 0.452 | 0.515 ± 0.193 $^{\gamma}$ |
| $N_2$ | 18 | 1.229 ± 0.298 $^{\gamma}$ | 0.439 ± 0.238 $^{\gamma}$ |
| Distant metastasis | | | |
| Negative | 4 | 1.531 ± 0.957 | 0.423 + 0.321 $^{\gamma}$ |
| No distant metastasis | 56 | 1.217 ± 0.442 | 0.472 + 0.226 $^{\gamma}$ |
| Tumour stage | | | |
| 0 | 6 | 1.531 ± 0.957 | 0.423 + 0.321 $^{\gamma}$ |
| I | 17 | 1.217 ± 0.372 $^{\gamma}$ | 0.490 + 0.235 $^{\gamma}$ |
| II | 4 | 1.050 ± 0.402 $^{\gamma}$ | 0.446 + 0.229 $^{\gamma}$ |
| III | 7 | 1.343 ± 0.382 | 0.487 + 0.114 $^{\gamma}$ |
| IV | 26 | 1.201 ± 0.524 | 0.465 + 0.247 $^{\gamma}$ |

*Note: N, number of patients; SD, standard deviation; $^{\alpha}$ No SD value was calculated for single case; $^{\beta}$ Age grouping was based on overall median age; $^{\gamma}$ Normal distribution data is assumed*

## Table 3.12: *t*-Test and ANOVA for means comparison

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics[α] (sig.) | Test[β] | Sig. (2-tailed) | Levene statistics[α] (sig.) | Test[β] | Sig. (2-tailed) |
| Gender | 0.765 | *t*-Test | 0.862 | 0.858 | *t*-Test | 0.424 |
| Age group | 0.068 | *t*-Test | 0.140 | 0.218 | *t*-Test | 0.966 |
| Anatomic sites[γ] | 0.089 | ANOVA | 0.241 | 0.373 | ANOVA | 0.273 |
| Disease outcome | 0.034 | *t*-Test | 0.212 | 0.756 | *t*-Test | 0.937 |
| Tumour size[γ] | 0.014 | ANOVA | 0.016* | 0.487 | ANOVA | 0.234 |
| Regional lymph nodes | 0.067 | ANOVA | 0.668 | 0.424 | ANOVA | 0.831 |
| Distant metastasis | 0.019 | *t*-Test | 0.214 | 0.386 | *t*-Test | 0.681 |
| Tumour stage | 0.227 | ANOVA | 0.659 | 0.227 | ANOVA | 0.984 |

*Notes:*

*[α] - Levene's Test for Equality of Variances*

*[β] - Test for Equality of Means*

*[γ] - Cases less than two were excluded from analysis of variance (ANOVA)*

** Statistically significant at the 0.05 level*

## Table 3.13: Mann-Whitney and Kruskal Wallis for means comparison

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test[α] | Asymp. sig. | Test[α] | Asymp. sig. |
| Gender | Mann-Whitney | 0.923 | Mann-Whitney | 0.494 |
| Age group | Mann-Whitney | 0.258 | Mann-Whitney | 0.871 |
| Anatomic sites[β] | Kruskal-Wallis | 0.318 | Kruskal-Wallis | 0.136 |
| Disease outcome | Mann-Whitney | 0.312 | Mann-Whitney | 0.833 |
| Tumour size[β] | Kruskal-Wallis | 0.065 | Kruskal-Wallis | 0.237 |
| Regional lymph nodes | Kruskal-Wallis | 0.675 | Kruskal-Wallis | 0.877 |
| Distant metastasis | Mann-Whitney | 0.989 | Mann-Whitney | 0.577 |
| Tumour stage | Kruskal-Wallis | 0.798 | Kruskal-Wallis | 0.981 |

*Notes:*

*[α] - Test for Equality of Means*

*[β] - Cases less than two were excluded from Kruskal-Wallis Test*

### 3.4.3 DNA quality determination by PCR for β-globin detection

The first attempt successfully amplified this fragment in 52 out of 60 samples and human salivary gland DNA (positive control). Eight samples and 3T3 embryonic mouse fibroblasts (positive control), which were negative for β-globin were repeated for PCR and DNA extraction if required, as illustrated in Figure 2.5. Finally, all 60 samples were positive for β-globin gene and the template DNA were suitable to proceed to the next stage, which was PCR optimisation for L1 gene detection of HPV (Appendix 3.14). Unfortunately, 3T3 embryonic mouse fibroblasts failed to amplify the 268 bp fragment after several attempts and were removed as an alternative positive control. The human salivary gland DNA has remained a positive control for future down-stream analysis.

### 3.4.4 Summary of the PCR amplification of L1 HPV gene detections

The overall results for each sample (positive and negative separately) for L1 HPV gene detection was given in Appendix 3.14. The summary of the positive PCR amplification for L1 HPV gene is shown in Table 3.14.

**Table 3.14: PCR positivity for L1 HPV gene detection by PCR types**

| Type of PCR amplification | Expected amplicon sizes (% positivity) | | |
|---|---|---|---|
| | 450 bp | 190 bp | 140 bp |
| Independent PCR | 14[a] (23.3) | 6 (10.0) | 19 (31.7) |
| Semi-nested PCR | | 14 (23.3) | |
| Nested PCR | | | 14 (23.3) |
| Total sample positive for L1 HPV | 14 (23.3) | 20 (33.3) | 33 (55.0) |
| Total sample tested | 60 | 60 | 60 |

Note:
[a] - First round PCR amplicons (450 bp) as the template for either semi-nested or nested PCR amplifications

### 3.4.5 Purification of the PCR products

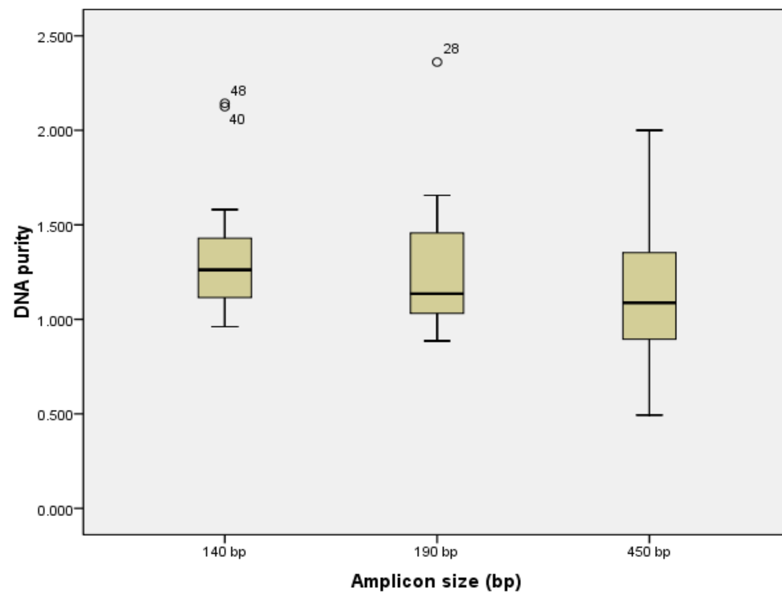
All 67 amplicons positive for L1 HPV gene were purified either directly using QIAquick PCR purification kit or QIAquick gel extraction kit (Appendix 15) following the criteria described in Materials and Methods. Each quantified amplicon was further diluted with sterile $dH_2O$ to the final concentration of 10 ng/µl (20 µl) prior to automated DNA sequencing. Means comparisons with regard to DNA purity and DNA yield of the purified PCR products are shown in Table 3.15. Statistical analyses for means comparison (Table 3.16 and 3.17), we concluded that DNA purity and DNA yield were comparable amongst the three different amplicon size. In addition, for QIAquick PCR Purification kit and QIAquick Gel Extraction kit comparison, the former was statistically better for DNA yield but the latter was better for DNA purity.

**Table 3.15: Means comparison of the purified amplicons and kit used**

| Parameters | *N* | Mean ± *SD* | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (ng/µl) |
| Amplicon size (*N* = 67) | | | |
| 140 bp | 33 | 1.305 ± 0.271 | 79.39 ± 44.58$^{\alpha}$ |
| 190 bp | 20 | 1.246 ± 0.349 | 98.85 ± 49.55$^{\alpha}$ |
| 450 bp | 14 | 1.147 ± 0.382$^{\alpha}$ | 57.14 ± 30.55 |
| PCR Purification Kit (*N* = 67) | | | |
| QIAquick PCR Purification | 35 | 1.215 ± 0.318$^{\alpha}$ | 103.29 ± 37.57$^{\alpha}$ |
| QIAquick Gel Extraction | 32 | 1.297 ± 0.324 | 55.69 ± 40.45 |

*Note: N, number of amplicons; SD, standard deviation;* $^{\alpha}$ *Normal distribution data is assumed*

**(A)**




**(B)**

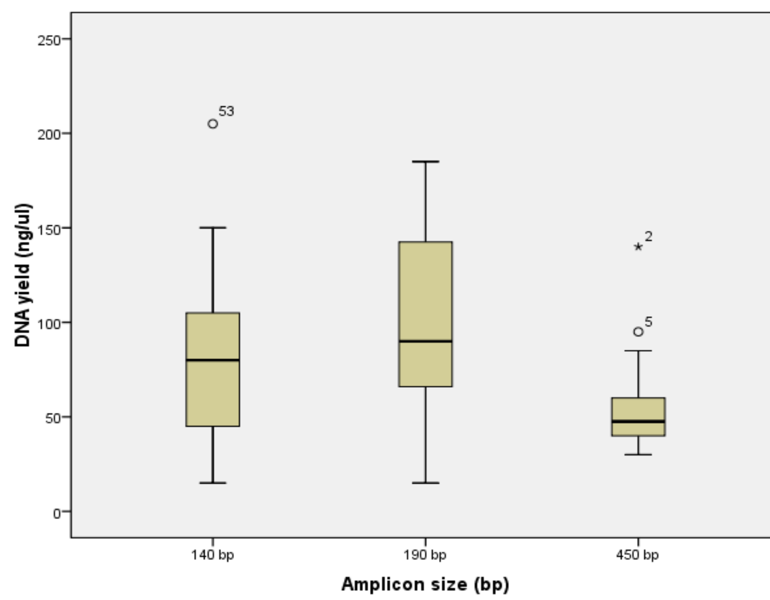
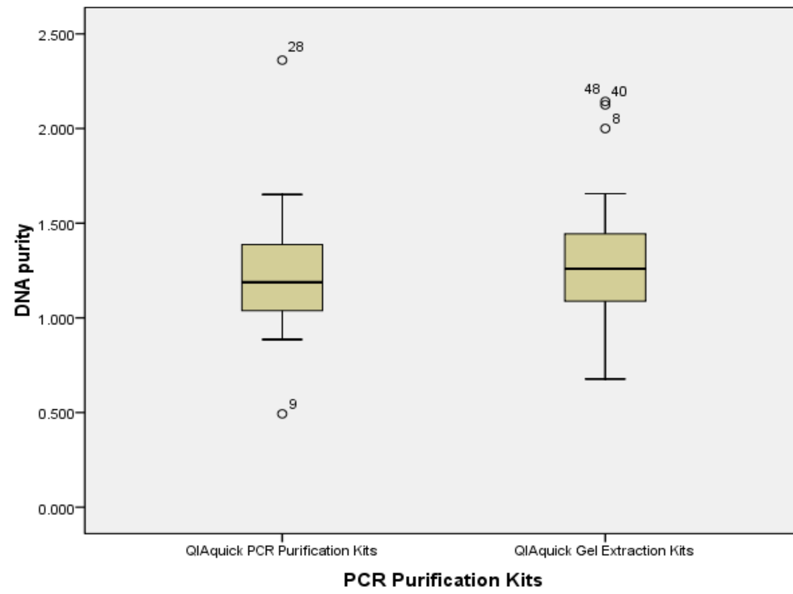



**Figure 3.10: DNA purity (A) and DNA yield (B) comparisons among PCR products**

**(A)**




**(B)**

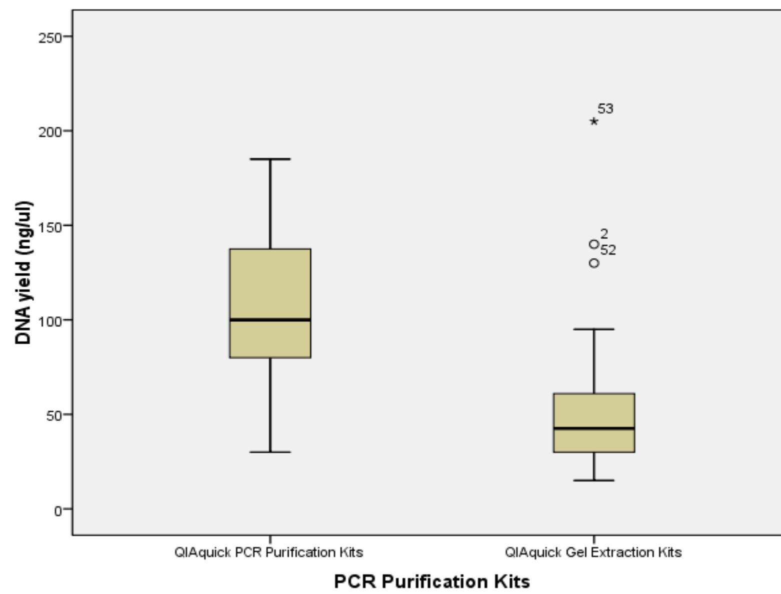



**Figure 3.11: DNA purity (A) and DNA yield (B) comparisons between purification kits**

**Table 3.16: *t*-Test and ANOVA**

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) |
| Amplicon size | 0.392 | ANOVA | 0.306 | 0.060 | ANOVA | 0.028* |
| DNA Purification Kits | 0.901 | *t*-Test | 0.302 | 0.540 | *t*-Test | 0.000** |

*Notes:*
*$^{\alpha}$ - Levene's Test for Equality of Variances*
*$^{\beta}$ - Test for Equality of Means*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of 190bp > 450 bp ($p<0.05$) and*
*QIAquick PCR Purification > QIAquick Gel Extraction ($p<0.001$)*

**Table 3.17: Mann-Whitney and Kruskal-Wallis Test**

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test$^{\alpha}$ | Asymp. sig. | Test$^{\alpha}$ | Asymp. sig. |
| Amplicon size | Kruskal-Wallis | 0.149 | Kruskal-Wallis | 0.034* |
| DNA Purification Kits | Mann-Whitney | 0.533 | Mann-Whitney | 0.000** |

*Notes:*
*$^{\alpha}$ - Test for Equality of Means*
*Cases less than two were excluded from Kruskal-Wallis Test*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of 190bp > 450 bp ($p<0.05$) and QIAquick PCR kits > QIAquick Gel kits ($p<0.001$)*

### 3.4.6 DNA sequencing of the PCR products

The diluted DNA of each amplicon was sequenced using both forward and reverse primers in separate sequencing reactions. The sample DNA sequencing results (MY11, forward primer and MY09, reverse primer) were visualised using Chromas Lite is shown in Figure 3.12. Both arrows show part of the overlapping DNA sequence and should be read according to their direction and in complement sequence such as (220 to

210; 3’- ACTGTCCATTA – 5’) from MY11 and its complementary sequence (67 to 76; 5’ – TGACAGGTAA – 3’) from MY09. The complementary sequence of the MY09 primer could be located at the end of MY11 primer DNA sequencing result and vice versa as shown in the schematic representation below (Figure 3.13). The DNA contig (the overlapping and non-overlapping DNA sequence including both primer sequences at the terminal of the sequence) will be formed according to expected amplicon size (bp). The raw data for the automated DNA sequencing of the purified amplicons are given in Appendix 16. The actual size of 56 out of 67 samples (83.6%) were successfully generated contigs for the actual size of the amplicons. Only the partial DNA sequences obtained in eleven amplicons (16.6%) due to only forward primer sequencing reactions were successful, therefore DNA contigs couldn’t be formed. Nevertheless, it did not affect further DNA sequences alignment

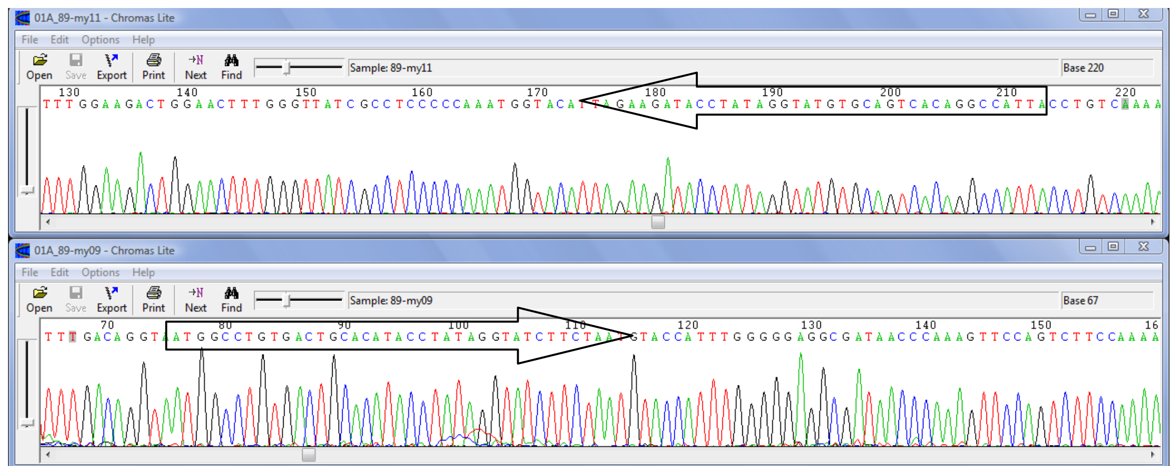



**Figure 3.12: Selected automated DNA sequencing results**

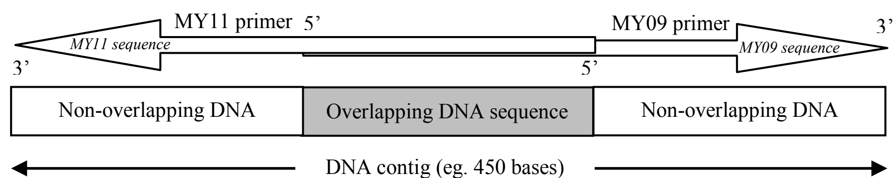



**Figure 3.13: Schematic representation of DNA contig formation**

### 3.4.7 DNA sequences alignment

The DNA contig obtained from each sample was individually compared with the sequences in the databases using Blastn (nr, non-redundant). The sample of DNA multiple sequence alignment carried out using known HPV6, 16, and 18 (Appendix 17). The results of all DNA sequences for the identification L1 alpha HPV via DNA sequences alignment are given in Appendix 18. The summary of the alpha HPV types detected is shown in Table 3.18 and Figure 3.14 in which 53.3% LR α-HPV (all HPV6) and 3.3% HR α-HPV were detected.

**Table 3.18: The summary of alpha HPV types detected via PCR and sequencing**

| Type of infection | HPV type | HPV type *N* (%) | α-HPV group *N* (%) |
|---|---|---|---|
| Single infection: | | | |
| Low risk α-HPV | HPV6 | 32 (53.3) | 32 (53.3) |
| High risk α-HPV | HPV16<br>HPV35 | 1 (1.7)<br>1 (1.7) | 2 (3.3) |
| | Total sample | 60 | 60 |

*N – number of sample*

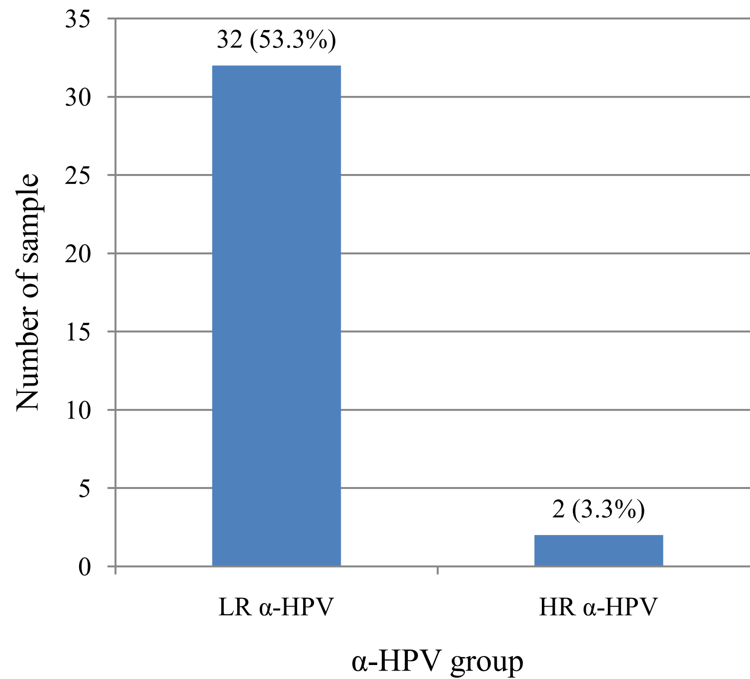



**Figure 3.14: LR α-HPV and HR α-HPV detected via PCR and DNA sequencing**

### 3.5 α-HPV GENOTYPING

The combination of SPF-DEIA (results shown in Appendix 19) and $SPF_{10}$-$LiPA_{25}$ (version 1) (results shown in Appendix 20) approaches for α-HPV genotyping was formally known as $SPF_{10}$-$LiPA_{25}$ (version 1). The interpretation sheet for α-HPV genotyping is given in Appendix 21. The cut-off point (borderline) for HPV positivity utilising SPF-DEIA was 0.531. It was found that 16 samples (26.7%) were positive for HPV as the first screening and eligible for the next $SPF_{10}$-$LiPA_{25}$ (version 1) for the HPV genotyping. The results of $SPF_{10}$-$LiPA_{25}$ (version 1), HPV genotyping for all 60 samples are summarised in Appendix 22. The summary of 16 α-HPV types (26.7%) positively detected is shown in Table 3.19. The single infection found for HPV types: 10.0% LR α-HPV (all HPV6) and 11.7% HR α-HPV were detected. One sample, #51 (1.7%) exhibited double infection of LR α-HPV and HR α-HPV types.

**Table 3.19: The summary of alpha HPV types detected by via $SPF_{10}$-$LiPA_{25}$ (version 1)**

| Mode of HPV infection | HPV type | HPV type *N* (%) | α-HPV group *N* (%) |
|---|---|---|---|
| N/I | N/I | 2 (3.3) | 2 (3.3) |
| Single infection: | | | |
| Low risk α-HPV | HPV6 | 6 (10.0) | 6 (10.0) |
| High risk α-HPV | HPV16 | 5 (8.3) | 7 (11.7) |
| | HPV18 | 1 (1.7) | |
| | HPV35 | 1 (1.7) | |
| Double infection: | | | |
| LR/HR α-HPV | HPV6/HPV16 | 1 (1.7) | 1 (1.7) |
| | Total sample | 60 | 60 |

*N – number of sample; N/I – not identified*

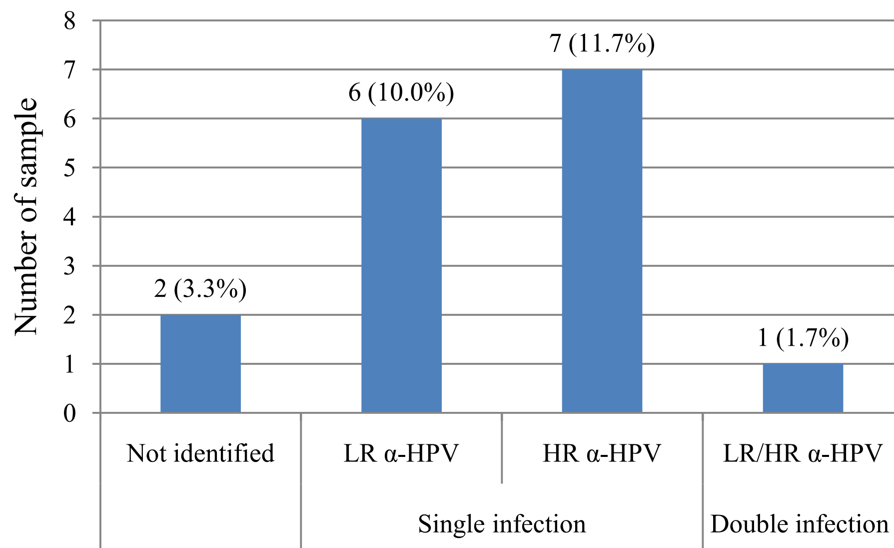



**Figure 3.15: LR α-HPV and HR α-HPV detected via $SPF_{10}$-$LiPA_{25}$ (version 1)**

The results from PCR and DNA sequencing and $SPF_{10}$-$LiPA_{25}$ for α-HPV genotyping of the same sample is shown in Appendix 23. We did not exclude the

possibility that more than one HPV types were present in the same sample, therefore we combined the results from both methods. The summary of the alpha HPV types detected is shown in Table 3.20. The single infection found for HPV types: 41.7% LR α-HPV (all HPV6) and 3.3% HR α-HPV were detected (Figure 3.16). Double infection was found in 15% of the samples including two unknown HPV types (N/I).

**Table 3.20: Alpha HPV types detected via the combination results of two methods**

| Mode of HPV infection | HPV type | HPV type *N* (%) | α-HPV group *N* (%) |
|---|---|---|---|
| Single infection: | | | |
| Low risk α-HPV | HPV6 | 25 ( 41.7) | 25 (41.7) |
| High risk α-HPV | HPV16<br>HPV35 | 1 (1.7)<br>1 (1.7) | 2 (3.3) |
| Double infection: | | | |
| LR/HR α-HPV | HPV6/HPV16<br>HPV6/HPV18<br>HPV6/ N/I | 6 (10.0)<br>1 (1.7)<br>2 (3.3) | 9 (15.0) |
| | Total sample | 60 | 60 |

*N – number of sample; N/I – α-HPV untypable*

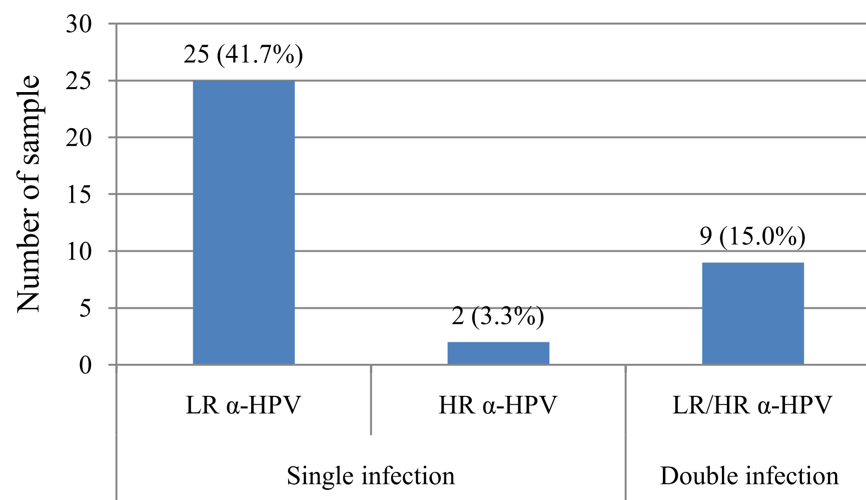



**Figure 3.16: LR α-HPV and HR α-HPV detected via both methods**

## 3.6 β-HPV GENOTYPING

The raw data of β-HPV genotyping for all 60 samples by using PM-PCR approaches are given in Appendix 24 and are summarised in Appendix 26. The interpretation sheet for β-HPV genotyping is given in Appendix 25. The summary of the alpha HPV types detected is shown in Table 3.21 and Figure 3.17. The single infection found for HPV types: 41.7% LR α-HPV (all HPV6) and 3.3% HR α-HPV were detected.

**Table 3.21: Beta HPV types detected via PM-PCR and RHA methods**

| Mode of HPV infection | HPV type | HPV type *N* (%) | β-HPV status *N* (%) |
|---|---|---|---|
| Single infection: | | | |
| | HPV15 | 1( 1.7) | 8 (13.3) |
| | HPV23 | 1 (1.7) | |
| | HPV36 | 1 (1.7) | |
| | HPV38 | 1 (1.7) | |
| | HPV76 | 1 (1.7) | |
| | HPV80 | 1 (1.7) | |
| | HPV93 | 2 (3.3) | |
| | N/I | 3 (5.0) | 3 (5.0) |
| Total (positive) | | | 11 (18.3) |
| | Total sample | 60 | 60 |

*N – number of sample; N/I – β-HPV untypable*

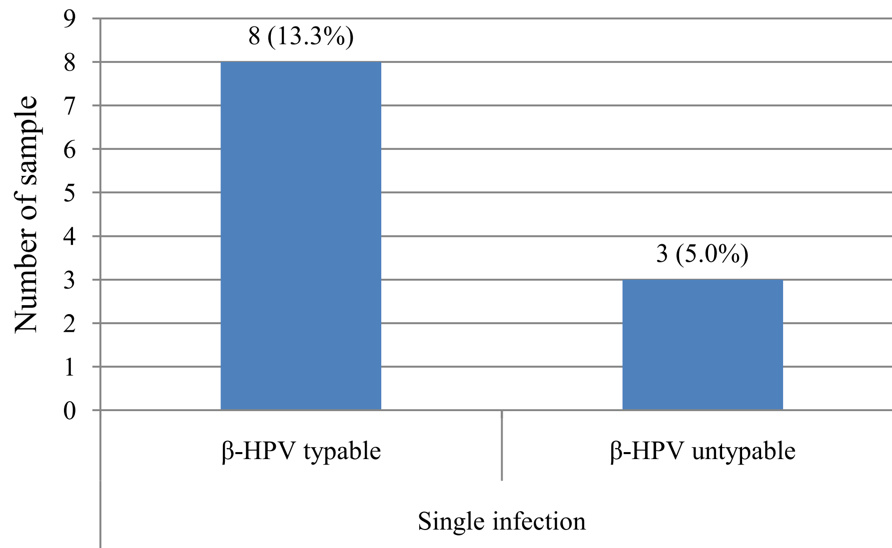



**Figure 3.17: The status of β-HPV types detected**

## 3.7 OVERALL HPV DETECTION

Similarly, (as previously described in 3.1.12) we did not exclude the possibility that more than one HPV types were present in the same sample, therefore we combined the results from α-HPV and β-HPV genotyping methods to obtain the full spectrum of infections which possibly existed within all the samples tested (Appendix 27). In total 40 out of 60 cases (66.7%) were positive for HPV in this cohort regardless of the type of HPV and the mode of infection (Figure 3.18 and Table 3.22). Figure 3.19 showing the percentage of HPV types distribution in which 11/56 (19.6%) each for HR α-HPV and β-HPV and 34/56 (60.7%) for LR α-HPV were detected in all samples by considering the mode of HPV infections. The three types of postulated HPV infection are illustrated in Figure 3.20, of which 27/40 (67.5%), 10/40 (25.0%) and 3/40 (7.5%), single, double and multiple infections, respectively. The breakdown (percentage) of each HPV type into HPV infection category is shown in Figure 3.21.

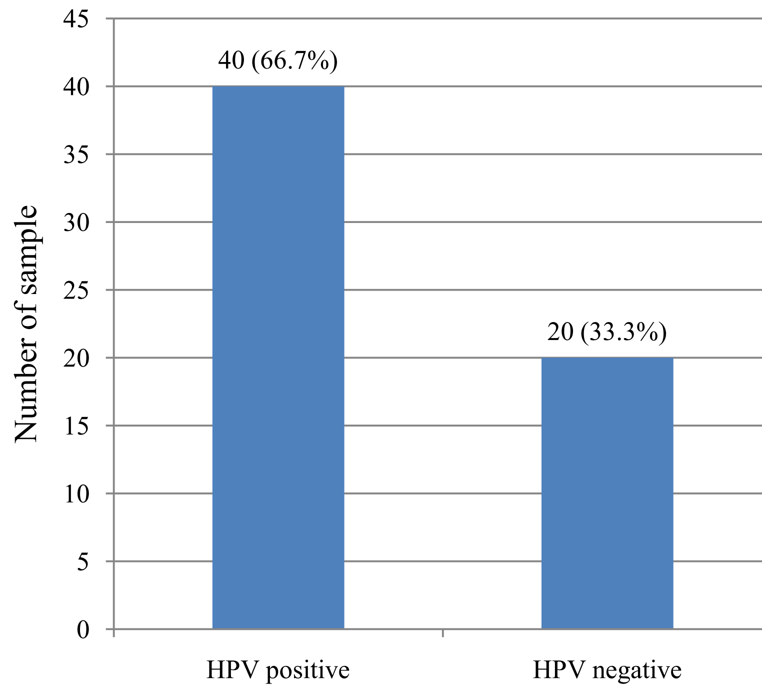



**Figure 3.18: The total of HPV positively detected in this pilot study**

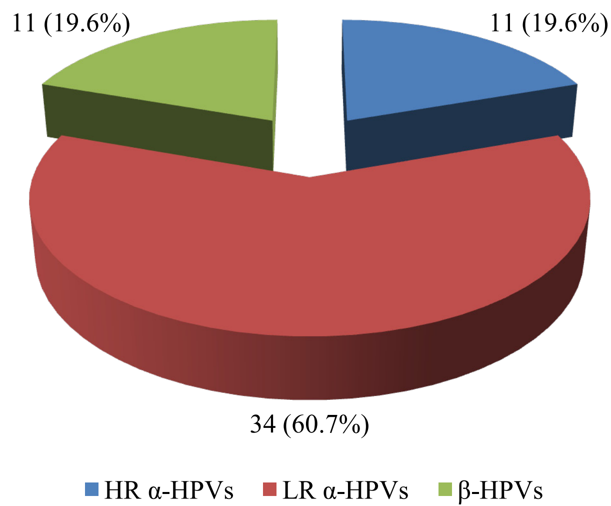



**Figure 3.19: The distribution of HPV types detected**
(*Note*: Multiple HPV types could exist in the same sample)

**Table 3.22: Overall HPV types detected by using the results of all methods**

| Mode of HPV infection | HPV type | HPV type *N* (%) | HPV group *N* (%) |
|---|---|---|---|
| **Single infection:** | | | |
| Low risk α-HPV | HPV6 | 21 (35.0) | 21 (35.0) |
| High risk α-HPV | HPV16 | 1 (1.7) | 2 (3.3) |
| | HPV35 | 1 (1.7) | |
| β-HPV | HPV15 | 1 (1.7) | 2 (3.3) |
| | HPV76 | 1 (1.7) | |
| β-HPV untypable | | 2 (3.3) | 2 (3.3) |
| | | | Total: 27 (45.0) |
| **Double infection:** | | | |
| LR/HR α-HPV | HPV6/HPV16 | 5 (8.3) | 6 (10.0) |
| | HPV6/HPV18 | 1 (3.3) | |
| LR α-HPV/β-HPV | HPV6/HPV36 | 1 (3.3) | 4 (6.7 ) |
| | HPV6/HPV38 | 1 (3.3) | |
| | HPV6/HPV80 | 1 (3.3) | |
| | HPV6/HPV93 | 1 (3.3) | |
| | | | Total: 10 (16.7) |
| **Multiple infection:** | | | |
| α-HPV/β-HPV | HPV6/HPV16/β-HPV untypable | 1 (3.3) | 3 ( 5.0) |
| | HPV6/α-HPV untypable/HPV23 | 1 (3.3) | |
| | HPV6/α-HPV untypable/HPV93 | 1 (3.3) | |
| | | | Total: 3 (5.0) |
| | Total (positive) | 40 | |
| | Percentage | 66.7 | |
| | Total sample | 60 | |

*N – number of sample; The percentage was calculated based on the total number of samples, 60*

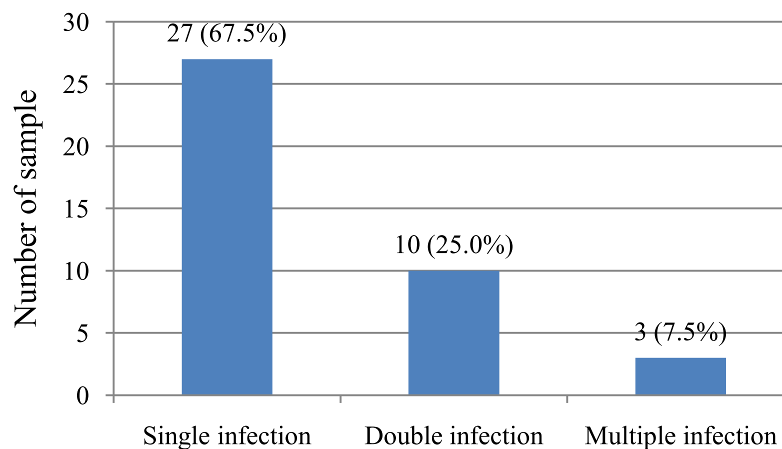



**Figure 3.20: The postulated mode of HPV infection**

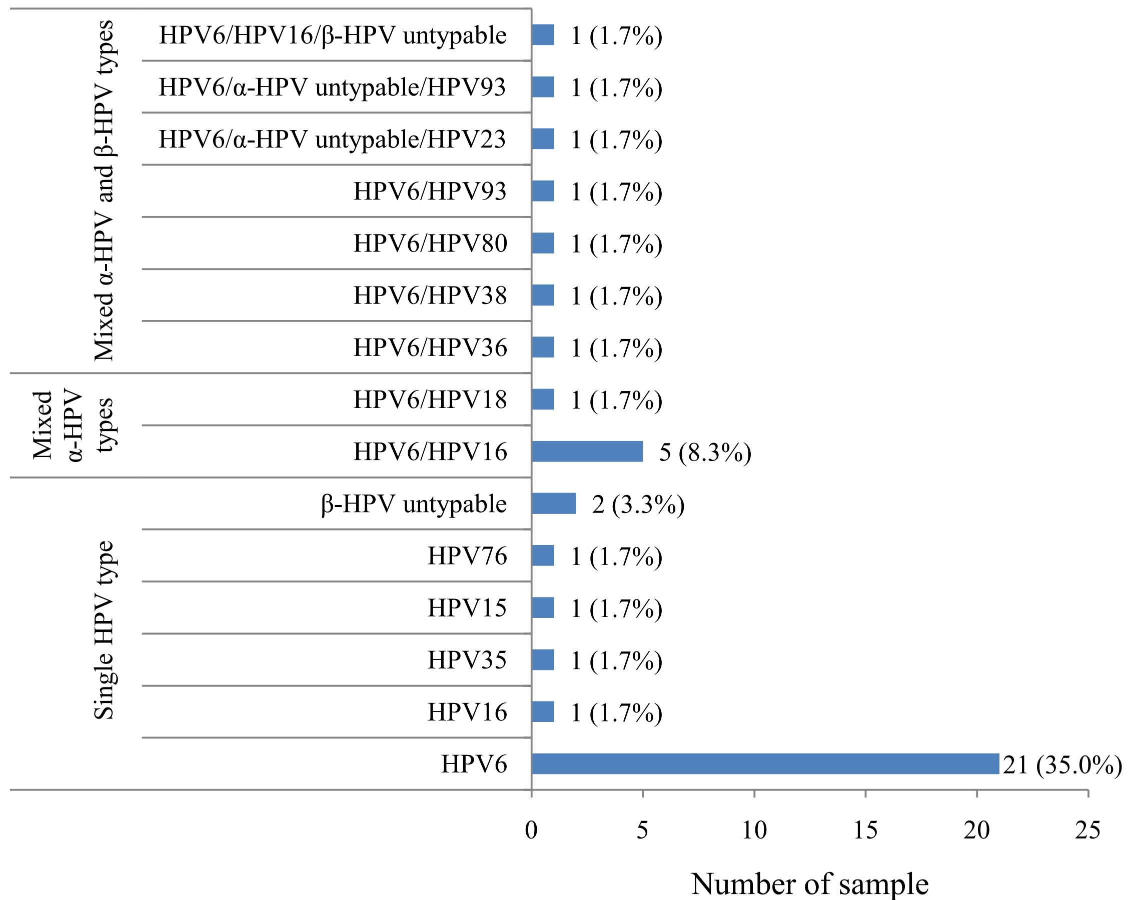



**Figure 3.21: The distribution of HPV types detected**

### 3.7.1 HPV prevalence and clinicopathological parameters

Most statistics books do not provide advice about how to decide whether a variable is at least approximately normal. SPSS recommends that we divide the skewness by its standard error. If the result is less than 2.5 (which is approximately the $p$=0.01 level) then skewness is *not* significantly different from normal. The set back of this method is that the standard error relies on the sample size and greatly influenced with large samples in which most variables would be found to be non normal (Coakes and Steed, 2001).

Instead of checking the skewness value, we could also compare the mean, median and mode values which can be obtained with the *Frequencies* command. If these values are approximately equal, then we can assume that the distribution is approximately normal. A simpler guideline is that if the skewness is less than plus or minus one (< +/-1.0) the variable is at least approximately normal (Hair et al., 1998; Morgan et al., 2004). Table 3.23 shows the determination of normally distributed data and the recommended statistical analyses for means comparison between/among categories/groups in each individual parameter.

**Table 3.23: Normally distributed data assumption among parameters**

| Parameter | *N* | *Sn* | *S.E.* | (-1/+1) | *Sn*/*S.E.* (<2.5) | Recommended statistics for means comparison |
|---|---|---|---|---|---|---|
| Total number of samples in each parameter, N = 60 | | | | | | |
| Gender | 2 | -0.206 | 0.309 | ND | -0.7 | *t*-Test |
| Age group | 2 | 0.068 | 0.309 | ND | 0.2 | *t*-Test |
| Disease outcome | 2 | 0.419 | 0.309 | ND | 1.4 | *t*-Test |
| Anatomic site | 10 | 1.504 | 0.309 | | 4.9 | Kruskal-Wallis |
| Tumour size | 4 | 0.649 | 0.309 | ND | 2.1 | ANOVA |
| Regional Lymph Nodes | 3 | 0.197 | 0.309 | ND | 0.6 | ANOVA |
| Distant Metastasis | 2 | -3.564 | 0.309 | | -11.5 | Mann-Whitney |
| Tumour stage | 4 | -0.361 | 0.314 | ND | -1.1 | ANOVA |
| Recurrence | 2 | 2.736 | 0.309 | | 8.9 | Kruskal-Wallis |
| Smoking | 2 | -0.842 | 0.314 | ND | -2.7 | *t*-Test |
| Alcohol | 2 | -2.156 | 0.314 | | -6.9 | Mann-Whitney |
| Smoking-Alcohol | 4 | -1.442 | 0.314 | | -4.6 | Kruskal-Wallis |
| Overall HPV positivity | 2 | -0.492 | 0.309 | ND | -1.6 | *t*-Test |
| α-HPV positivity | 2 | -0.137 | 0.309 | ND | -0.4 | *t*-Test |
| High risk HPV | 2 | 2.679 | 0.309 | | 8.7 | Mann-Whitney |
| Low risk HPV | 2 | -0.137 | 0.309 | ND | -0.4 | *t*-Test |
| β-HPV positivity | 2 | 1.679 | 0.309 | | 5.4 | Mann-Whitney |
| β-HPV types | >2 | 3.101 | 0.309 | | 10.0 | Kruskal-Wallis |
| p16 IHC | 2 | -1.411 | 0.309 | | -4.6 | Mann-Whitney |

Note: *n* - number of categories/groups; ND – normally distributed data is assumed; *Sn* – Skewness, *S.E.* – Skewness Standard Error

### 3.7.2 Correlation among demographic and clinical parameters associated with HPVs

Risk factors and demographic characteristics included gender, age group, alcohol and smoking exposure, tumour site, tumour stage, nodal involvement and disease recurrence. Unadjusted associations between categorical variables were tested with either the Pearson chi-square test of Fisher's exact test for small sample sizes. We also tested for possible interactions among risk factors. Tests for interactions were generated using both logistic regression and proportional hazard regression methods. Multivariate unconditional logistic regression was used to examine the association between dichotomous dependent variables and risk factors for oral cancer (gender, age group, alcohol and smoking). Odds ratio (OR) and 95% confidence interval (CI) were calculated to measure the magnitude of the associations.

**Table 3.24: Demographic and risk factors of oral cancer by HPV status**

| Characteristics | *N* | HPV-, *N*=20 (%) | HPV+, *N*=40 (%) | OR (95% CI) | *p* value |
|---|---|---|---|---|---|
| Gender | | | | | |
| Female$^{\alpha}$ | 33 | 9 (45.0) | 18 (45.0) | 1.0 | 0.090 |
| Male | 27 | 11 (55.0) | 22 (55.0) | 2.0 (0.9 – 4.5) | |
| Age group (range, 36-97y) | | | | | |
| 36-65y$^{\alpha}$ | 31 | 12 (60.0) | 19 (47.5) | 1.0 | 0.213 |
| 66-97y | 29 | 8 (40.0) | 21 (52.5) | 1.6 (0.8 – 3.3) | |
| Smoking | | | | | |
| No$^{\alpha}$ | 20 | 4 (20.0) | 16 (40.0) | 1.0 | 0.013* |
| Yes | 40 | 16 (80.0) | 24 (60.0) | 4.0 (1.3 – 12.0) | |
| Alcohol | | | | | |
| No$^{\alpha}$ | 10 | 2 (10.0) | 8 (20.0) | 1.0 | 0.080 |
| Yes | 50 | 18 (90.0) | 32 (80.0) | 4.0 (0.8 – 19.0) | |
| Smoking-Alcohol | | | | | 0.053 |
| NSND$^{\alpha}$ | 5 | 1 (5.0) | 4 (10.0) | 1.0 | |
| NSD | 5 | 1 (5.0) | 4 (10.0) | 4.0 (0.4 – 36.0) | |
| SND | 15 | 3 (15.0) | 12 (30.0) | 4.0 (0.4 – 36.0) | |
| SD | 35 | 15 (75.0) | 20 (50.0) | 4.0 (1.1 – 14.0) | |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level; $^{\alpha}$ - reference for odds ratio determination

**Table 3.25: Clinicopathologic characteristics of oral cancer by HPV status**

| Characteristics | *N* | HPV-, *N*=20 (%) | HPV+, *N*=40 (%) | OR (95% CI) | *p* value |
|---|---|---|---|---|---|
| Anatomic site | | | | | |
| Buccal mucosa[α] | 1 | 0 (0) | 1 (2.5) | 1.0 | 0.740 |
| Floor of the mouth | 10 | 6 (30.0) | 4 (10.0) | - | |
| Alveolus | 4 | 1 (5.0) | 3 (7.5) | 0.7 (0.2 – 2.4) | |
| Soft palate | 3 | 0 (0) | 3 (7.5) | 3.0 (0.3 – 28.8) | |
| Pharynx | 1 | 0(0) | 1 (2.5) | - | |
| Lower lip | 1 | 0 (0) | 1 (2.5) | - | |
| Retromolar pad | 9 | 1 (5.0) | 8 (20.0) | - | |
| Supraglottis | 1 | 0(0) | 1 (2.5) | 8.0 (1.0 – 64.0) | |
| Tongue | 29 | 12 (60.0) | 17 (42.5) | - | |
| Tonsil | 1 | 0(0) | 1(2.5) | 1.4 (0.7 – 3.0) | |
| Tumour size | | | | | |
| Negative[α] | 2 | 0 (0) | 2 (5.0) | 1.0 | 0.835 |
| $T_1$ (<2 cm) | 24 | 11 (55.0) | 13 (32.5) | - | |
| $T_2$ (2-4 cm) | 19 | 7 (35.0) | 12 (30.0) | 1.2 (0.5 – 2.6) | |
| $T_3$ (>4 cm) | 1 | 0 (0) | 1 (2.5) | 1.7 (0.7 – 4.3) | |
| $T_4$ (>4 cm) | 14 | 2 (10.0) | 12 (30.0) | - | |
| Regional lymph nodes | | | | | |
| Negative[α] | 4 | 1 (5.0) | 3 (7.5) | 1.0 | 0.373 |
| $N_0$ | 28 | 10 (50.0) | 18 (45.0) | 3.0 (0.3 – 29.0) | |
| $N_1$ | 10 | 5 (25.0) | 5 (12.5) | 1.8 (0.8 – 3.9)) | |
| $N_2$ | 18 | 4 (20.0 | 14 (35.0) | 1.0 (0.3 – 3.5) | |
| Distant metastasis | | | | | |
| Negative[α] | 4 | 1 (5.0) | 3 (7.5) | 1.0 | 0.341 |
| No distant metastasis | 56 | 19 (95.0) | 37 (92.5) | 3.0 (0.3 – 28.8) | |
| Tumour stage | | | | | |
| 0[α] | 6 | 2 (10.0) | 4 (10.0) | 1.0 | 0.933 |
| I | 17 | 9 (45.0) | 8 (20.0) | 2.0 (0.4 – 11.0) | |
| II | 4 | 0 (0) | 4 (10.0) | 0.9 (0.3 – 2.3) | |
| III | 7 | 3 (15.0) | 4 (10.0) | - | |
| IV | 26 | 6 (30.0) | 20 (50.0) | 1.3 (0.3 – 6.0) | |
| Disease outcome | | | | | |
| Alive | 24 | 6 (30.0) | 18 (45.0) | 3.0 (1.2 – 7.6) | 0.020* |
| Dead[α] | 36 | 14 (70.0) | 22 (55.0) | 1.0 | |
| Disease status | | | | | |
| No recurrence[α] | 54 | 19 (95.0) | 35 (87.5) | 1.0 | 0.032* |
| Recurrence | 6 | 1 (5.0) | 5 (12.5) | 1.8 (1.0 – 3.2) | |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level; [α] - reference for odds ratio determination

## Table 3.26: The distribution of HPV types within demographic characteristics

| | HR α-HPV types (%) | | | | LR α-HPV (%) | β-HPV types (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HPV16 | HPV18 | HPV35 | Untypable | HPV6 | HPV15 | HPV23 | HPV36 | HPV38 | HPV76 | HPV80 | HPV93 | Untypable |
| **Gender:** | | | | | | | | | | | | | |
| Female | 4 (57.1) | - | - | 1 (50.0) | 17 (50.0) | 1 (100.0) | - | 1 (100.0) | 1 (100.0) | - | 1 (100.0) | 2 (100.0) | 1 (33.3) |
| Male | 3 (42.9) | 1 (100.0) | 1 (100.0) | 1 (50.0) | 17 (50.0) | - | 1 (100.0) | - | - | 1 (100.0) | - | - | 2 (66.7) |
| **Age group:** | | | | | | | | | | | | | |
| 36 – 65y | 3 (42.9) | 1(100.0) | 1 (100.0) | 1 (50.0) | 16 (47.1) | - | 1 (100.0) | - | 1 (100.0) | - | - | - | 1 (33.3) |
| 66 – 97y | 4 (57.1) | - | - | 1 (50.0) | 18 (52.9) | 1 (100.0) | - | 1 (100.0) | - | 1 (100.0) | 1 (100.0) | 2 (100.0) | 2 (66.7) |
| **Smoking:** | | | | | | | | | | | | | |
| No | 3 (42.9) | - | 1 (100.0) | - | 15 (44.1) | - | - | 1 (100.0) | 1 (100.0) | - | - | - | 1(33.3) |
| Yes | 4 (57.1) | 1 (100.0) | - | 2 (100.0) | 19 (55.9) | 1 (100.0) | 1 (100.0) | - | - | 1 (100.0) | 1 (100.0) | 2 (100.0) | 2 (66.7) |
| **Alcohol:** | | | | | | | | | | | | | |
| No | 1 (14.3) | 1 (100.0) | - | - | 7 (20.6) | - | - | 1 (100.0) | - | 1 (100.0) | 1 (100.0) | - | 1 (33.3) |
| Yes | 6 (85.7) | - | 1 (100.0) | 2 (100.0) | 27 (79.4) | 1 (100.0) | 1 (100.0) | - | 1 (100.0) | - | - | 2 (100.0) | 2 (66.7) |
| **Smoking-Alcohol:** | | | | | | | | | | | | | |
| NSND | 1 (14.3) | - | - | - | 4 (11.8) | - | - | 1 (100.0) | - | - | - | - | 1 (33.3) |
| NSD | 2 (28.6) | - | 1 (100.0) | - | 11 (32.4) | - | - | - | 1 (100.0) | - | - | - | - |
| SND | - | 1 (100.0) | - | - | 3 (8.8) | - | - | - | - | 1 (100.0) | 1 (100.0) | - | - |
| SD | 4 (57.1) | - | - | 2 (100.0) | 16 (47.1) | 1 (100.0) | 1 (100.0) | - | - | - | - | 2 (100.0) | 2 (66.7) |
| **Disease status:** | | | | | | | | | | | | | |
| No recurrence | 5 (71.4) | 1 (100.0) | 1 (100.0) | 2 (100.0) | 29 (85.3) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 2 (100.0) | 3 (100.0) |
| Recurrence | 2 (28.6) | - | - | - | 5 (14.7) | - | - | - | - | - | - | - | - |
| **Disease outcome:** | | | | | | | | | | | | | |
| Alive | 5 (71.4) | 1 (100.0) | 1 (100.0) | 1 (50.0) | 21 (61.8) | - | - | - | - | - | - | 2 (100.0) | - |
| Dead | 2 (28.6) | - | - | 1 (50.0) | 13 (38.2) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | - | 3 (100.0) |
| **Subtotal** | **7 (63.6)** | **1 (9.1)** | **1 (9.1)** | **2 (18.2)** | **34 (100.0)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **2 (18.2)** | **3 (27.3)** |
| **TOTAL[α] (*N* = 56)** | **11 (19.6)** | | | | **34 (60.7)** | **11 (19.6)** | | | | | | | |

Note:

[α] - Total number of HPV types by considering the mode of HPV infections

## Table 3.27: The distribution of HPV types within clinicopathological parameters

| | HR α-HPV types (%) | | | | LR α-HPV (%) | β-HPV types (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HPV16 | HPV18 | HPV35 | Untypable | HPV6 | HPV15 | HPV23 | HPV36 | HPV38 | HPV76 | HPV80 | HPV93 | Untypable |
| **Anatomic sites:** | | | | | | | | | | | | | |
| Buccal mucosa | - | - | - | - | 1 (2.9) | - | - | - | - | - | - | - | - |
| Floor of the mouth | - | - | - | 1 (50.0) | 4 (11.8) | - | - | - | - | - | - | 2 (100.0) | - |
| Alveolus | 1 (14.3) | - | - | - | 3 (8.8) | - | - | 1 (100.0) | - | - | - | - | - |
| Soft palate | 1 (14.3) | - | - | - | 2 (2.9) | - | - | - | - | 1 (100.0) | - | - | - |
| Pharynx | - | - | - | - | 1 (2.9) | - | - | - | - | - | - | - | - |
| Lower lip | - | - | - | - | 1 (2.9) | - | - | - | - | - | - | - | - |
| Retromolar pad | 2 (28.6) | - | - | - | 7 (20.6) | 1 (100.0) | - | - | - | - | - | - | 1 (33.3) |
| Supraglottis | - | - | - | - | 1 (2.9) | - | - | - | - | - | - | - | - |
| Tongue | 3 (42.9) | 1 (50.0) | 1 (100.0) | 1 (50.0) | 15 (44.1) | - | 1 (100.0) | - | 1 (100.0) | - | 1 (100.0) | - | 1 (33.3) |
| Tonsil | - | - | - | - | - | - | - | - | - | - | - | - | 1 (33.3) |
| **Tumour size:** | | | | | | | | | | | | | |
| Negative | - | - | - | - | 1 (2.9) | - | - | - | - | 1 (100.0) | - | - | - |
| T1 | 4 (57.1) | 1 (100.0) | - | 1 (50.0) | 13 (38.2) | - | - | - | 1 (100.0) | - | - | 2 (100.0) | - |
| T2 | 2 (28.6) | - | 1 (100.0) | - | 8 (23.5) | 1 (100.0) | - | - | - | - | - | - | 2 (66.7) |
| T3 | - | - | - | - | - | - | - | - | - | - | - | - | 1 (33.3) |
| T4 | 1 (14.3) | - | - | 1 (50.0) | 12 (35.3) | - | 1 (100.0) | 1 (100.0) | - | - | 1 (100.0) | - | - |
| **Regional lymph nodes:** | | | | | | | | | | | | | |
| Negative | - | 1 (100.0) | - | - | 2 (5.9) | - | - | - | - | 1 (100.0) | - | - | - |
| N0 | 5 (71.4) | - | 1 (100.0) | - | 15 (44.1) | 1 (100.0) | - | 1 (100.0) | - | - | - | - | 1 (33.3) |
| N1 | - | - | - | 2 (100.0) | 4 (11.8) | - | 1 (100.0) | - | - | - | - | 2 (100.0) | 1 (33.3) |
| N2 | 2 (28.6) | - | - | - | 13 (38.2) | - | - | - | 1 (100.0) | - | 1 (100.0) | - | 1 (33.3) |
| **Distant metastasis:** | | | | | | | | | | | | | |
| Negative | - | 1 (100.0) | - | - | 2 (5.9) | - | - | - | - | 1 (100.0) | - | - | - |
| No distant metastasis | 7 (100.0) | - | 1 (100.0) | 2 (100.0) | 32 (94.1) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | - | 1 (100.0) | 2 (100.0) | 3 (100.0) |
| **Tumour stage:** | | | | | | | | | | | | | |
| 0 | - | 1 (100.0) | - | - | 3 (8.8) | - | - | - | - | 1 (100.0) | - | - | - |
| I | 2 (28.6) | - | - | - | 8 (23.5) | - | - | - | - | - | - | - | - |
| II | 2 (28.6) | - | - | - | 3 (8.8) | - | - | - | - | - | - | - | 1 (33.3) |
| III | - | - | - | 1 (50.0) | 4 (11.8) | - | - | - | - | - | - | 2 (100.0) | - |
| IV | 3 (42.8) | - | 1 (100.0) | 1 (50.0) | 16 (47.1) | 1 (100.0) | 1 (100.0) | 1 (100.0) | 1 (100.0) | - | 1 (100.0) | - | 2 (66.7) |
| **Subtotal** | **7 (63.6)** | **1 (9.1)** | **1 (9.1)** | **2 (18.2)** | **34 (100.0)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **1 (9.1)** | **2 (18.2)** | **3 (27.3)** |
| **TOTAL (*N* = 56)** | **11 (19.6)** | | | | **34 (60.7)** | **11 (19.6)** | | | | | | | |

# Table 3.28: The correlations between demographic/clinical parameters and HPV status

| Parameter 1 | Parameter 2 | Pearson Correlation | | Spearman's rho | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Correlation coefficient | Sig. (2-tailed) |
| **Within HPV:** | | | | | |
| HPV status | α-HPV status | 0.809** | 0.000 | 0.809** | 0.000 |
| | HR-HPV status | 0.335** | 0.009 | 0.335** | 0.009 |
| | LR-HPV status | 0.809** | 0.000 | 0.809** | 0.000 |
| | HPV16 status | 0.257* | 0.047 | 0.257* | 0.047 |
| | β-HPV status | 0.335** | 0.009 | 0.335** | 0.009 |
| α-HPV status | HR-HPV status | 0.414** | 0.001 | 0.414** | 0.001 |
| | LR-HPV status | 0.864** | 0.000 | 0.846** | 0.000 |
| | HPV16 status | 0.318* | 0.013 | 0.318* | 0.013 |
| HR-HPV status | HPV16 status | 0.767** | 0.000 | 0.767** | 0.000 |
| **Between HPV and demographic/clinical parameters:** | | | | | |
| α-HPV status | Smoking-Alcohol | -0.262* | 0.043 | 0.262* | 0.043 |
| | Smoking | -0.333** | 0.009 | -0.333** | 0.009 |
| β-HPV status | Disease outcome (Dead-Alive) | -0.404** | 0.001 | -0.404** | 0.001 |
| LR-HPV status | Smoking | -0.262* | 0.043 | -0.262* | 0.043 |
| Recurrence | No association found | - | - | - | - |
| **Within demographic and clinical parameters:** | | | | | |
| Disease outcome (Dead-Alive) | Tumour size | -0.268* | 0.038 | -0.344** | 0.007 |
| | Tumour stage | -0.270* | 0.037 | -0.254 | 0.051 |
| | Smoking-Alcohol | 0.361** | 0.005 | 0.288* | 0.026 |
| | Alcohol | 0.365** | 0.004 | 0.365** | 0.004 |
| Tumour size | Tumour stage | 0.666** | 0.000 | 0.676** | 0.000 |
| | Regional lymph nodes | 0.286* | 0.027 | 0.352** | 0.006 |
| | Distant metastasis | 0.333** | 0.009 | 0.368** | 0.004 |
| Tumour stage | Regional lymph nodes | 0.488** | 0.000 | 0.479** | 0.000 |
| | Distant metastasis | 0.442** | 0.000 | 0.441** | 0.000 |
| Regional lymph nodes | Distant metastasis | 0.468** | 0.000 | 0.464** | 0.000 |
| | Anatomic site | 0.347** | 0.007 | 0.376** | 0.003 |
| Smoking-Alcohol | Alcohol | 0.870** | 0.000 | 0.729** | 0.000 |
| | Smoking | 0.625** | 0.000 | 0.749** | 0.000 |
| Smoking | Age group | 0.259* | 0.045 | 0.259* | 0.045 |
| Recurrence | No association found | - | - | - | - |

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

### 3.7.3 Preliminary conclusions

The demographic, risk factor and clinicopathological characteristics of oral cancer by HPV status are given in Table 3.24 and Table 3.25. It was found that HPV status was significantly associated with an increase in relative risk compared to the reference group, smokers [OR=4.0(1.3–12.0), $p<0.05$], survived patient [OR=3.0(1.2–7.6), $p<0.05$] and patients with disease recurrence [OR=1.8(1.0–3.2), $p<0.05$].

Table 3.26 and Table 3.27 illustrate the distribution of HPV types across demographic and clinical characteristics. Generally, it was observed that the distribution of HR-HPV types and LR-HPV types was nearly comparable across gender and age group. Both HPV risk groups followed a similar pattern in smokers and drinkers. It was found that both groups were identified in smokers compared to non-smokers. Even significantly higher number of both HPV risk groups detected among drinkers compared to non-drinkers. Similarly, SD and NSD were the two groups which demonstrated significantly higher HPV types observed and this was consistent in both HPV risk groups This pattern was also true in the number of patients with no disease recurrence for both groups. More female and older patients were identified positive for β-HPV types. A similar pattern of β-HPV types distribution was observed as was previously described for α-HPV groups (HR-HPV and LR-HPV) in smokers, drinkers, SD and no disease recurrence groups. More living patients were positive for both α-HPV groups but this was not the case for β-HPV types.

Generally, a very heterogeneous distribution of all HPV types was observed and again predominantly with HPV6 in most tumour sizes but typically demonstrated that

the frequency of HPV16 detected decreases as the tumour size increases. Anatomically, none of HR-HPV and β-HPV types were detected in buccal mucosa, pharynx, lower lip and supraglottis. None of the HPV types were identified in tonsil except in one case with untypable β-HPV. Tongue was the site where most of the different HPV types were detected and predominantly HPV6, 15/34 (44.1%). As it was absent in tonsil, HPV6 was the only candidate for LR-HPV found in all anatomic sites studied. An uneven distribution of HPV16 and other β-HPV types was observed but confined within alveolus, soft palate, retromolar pad and tongue.

The HPV type distribution in regional lymph nodes and tumour size relatively exhibited similar pattern particularly for HR-HPV and LR-HPV. Higher percentage of both of HPV risk groups were observed at tumour size less than 2-cm and no regional lymph nodes metastasis. As was previously described in disease recurrence, an almost similar distribution pattern of all HPV types was observed in the absent of distant metastasis. Nearly fifty percent of HR-HPV or LR-HPV were detected at very late tumour stage (Stage IV) and an even higher proportion of β-HPV types were observed in this group.

Correlation analyses were carried out to search for any existing association in HPV positivity, demographic and clinical parameters using Pearson's correlation and Spearman's correlation, rho in this cohort. A statistically significant association observed was grouped into three, within HPV status, between HPV status and demographic/clinical parameters and within demographic/clinical parameters (Table 3.28). Any significant associations (positive or negative) were transformed into schematic representation for better visualisation of the correlation as shown in Figure

3.22. We have to acknowledge that a more complex association exists involving multifactorial causes once dealing with patient disease outcome. We are fully aware of all the difficulties and constrains in obtaining full historical data especially related to retrospective study. Therefore, we tried our best to make full use of all the accessible data to assist in the correct interpretation of the results.

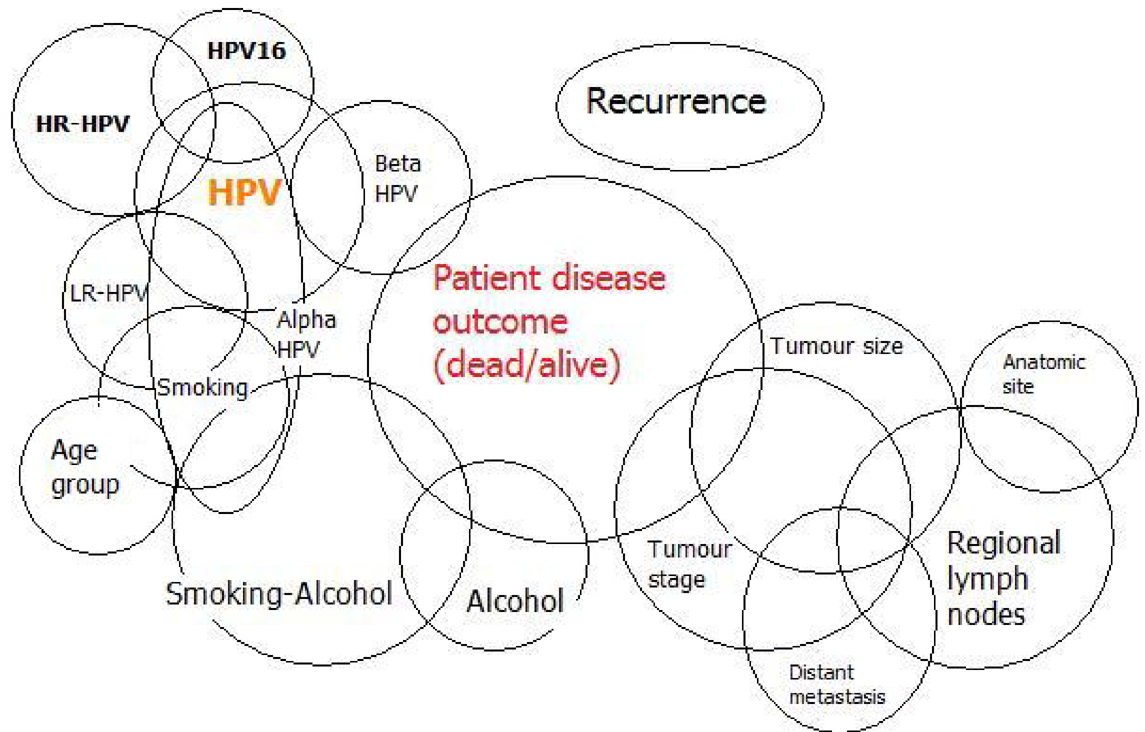



**Figure 3.22: Schematic representation of correlation plot amongst parameters.** Overlapping circle means a correlation exists. All parameters compared exhibit positive correlation except for an association between tumour size, tumour stage and β-HPV status towards patient disease outcome, LR-HPV status with smoking, α-HPV with smoking and smoking-alcohol which are negative as shown in Table 3.28. The size of the circle does not indicate the strength of the association.

For patient disease outcome, initially we set code '0' for dead and code '1' for alive for data entry in SPSS. Any negative association to another parameter will be interpreted as an increasing number of deceased patients. For instance, tumour size and tumour stage were negatively associated with patient disease outcome. With an increasing number of patients from $T_0$ to $T_4$ and tumour stage from stage 0 to IV, it is

more likely that we observe an increasing number of deceased patients as well. Since there are no direct associations between regional lymph nodes, distant metastasis and anatomic site with patient disease outcome, we presume these were negative, based on their positively associated with tumour size and tumour stage. For anatomic site, we gave a code in ascending order from ‘0’ for buccal mucosa to ‘9’ for tonsil for data entry in SPSS. Thus, we presume that more samples taken from the tongue were associated with an increasing number of deceased patients.

The positive correlation between alcohol and smoking-alcohol (increasing number of patient in SD group) with patient disease outcome appeared to be a controversial ‘protective effect’ of alcohol and smoking. Since we have not seen any association related to disease recurrence, this factor was put as a separate entity. To note, the above assumption was based on the preliminary small size of the cohort. Patient disease outcome was negatively associated with β-HPV types. In contrast, no direct link was observed either HR-HPV or HPV16 and patient disease outcome. Like anatomic site, which exhibited single association, more elderly patients were identified as the smokers.

## 3.8 p16 ROLES IN ORAL CANCER

### 3.8.1 Clinical data

The cohort of 60 patients was previously mentioned in section 3.1.1. The preparation of the tissue specimens was as described in section 2.2.6. Eight to ten individual sections mounted onto the labelled slides were prepared for each sample.

### 3.8.2 Semi-quantitative scoring for p16 immunohistochemistry

All p16 IHC slides were semi-quantitatively assessed as described in the appropriate part of Chapter 2. A representative of the tissue specimens demonstrating negative and positive staining with the p16 antibody are as shown in Figure 3.23. The staining intensity was included for p16 positivity determination based on a scale of 1+, 2+ and 3+ as depicted in Figure 3.24. Strong nuclear and/or cytoplasmic staining was considered positive. We commonly observed strong cytoplasmic staining compared to nuclear staining and this was limited to the tumour cells. Figure 3.24 (A) shows a typical pattern of p16 positive staining (only cytoplasmic staining not nuclear staining) was predominantly at basal and extending to a suprabasal region of the epithelium cells. Focal and basal positive staining appeared more frequent in our samples. Another additional feature that was observed i.e. homogenous (Figure 3.24 (C)) or heterogeneous stain and in most cases the former tends to be considered as diffuse. The latter, on the other hand, was more common at the invading area of SCC. Before statistical analysis was performed, the p16 positive distribution was converted to a positive and negative score. Although, positive staining of non-tumour cells, for instance epithelia and multinucleated giant cells were occasionally visualised, these were excluded as positive.

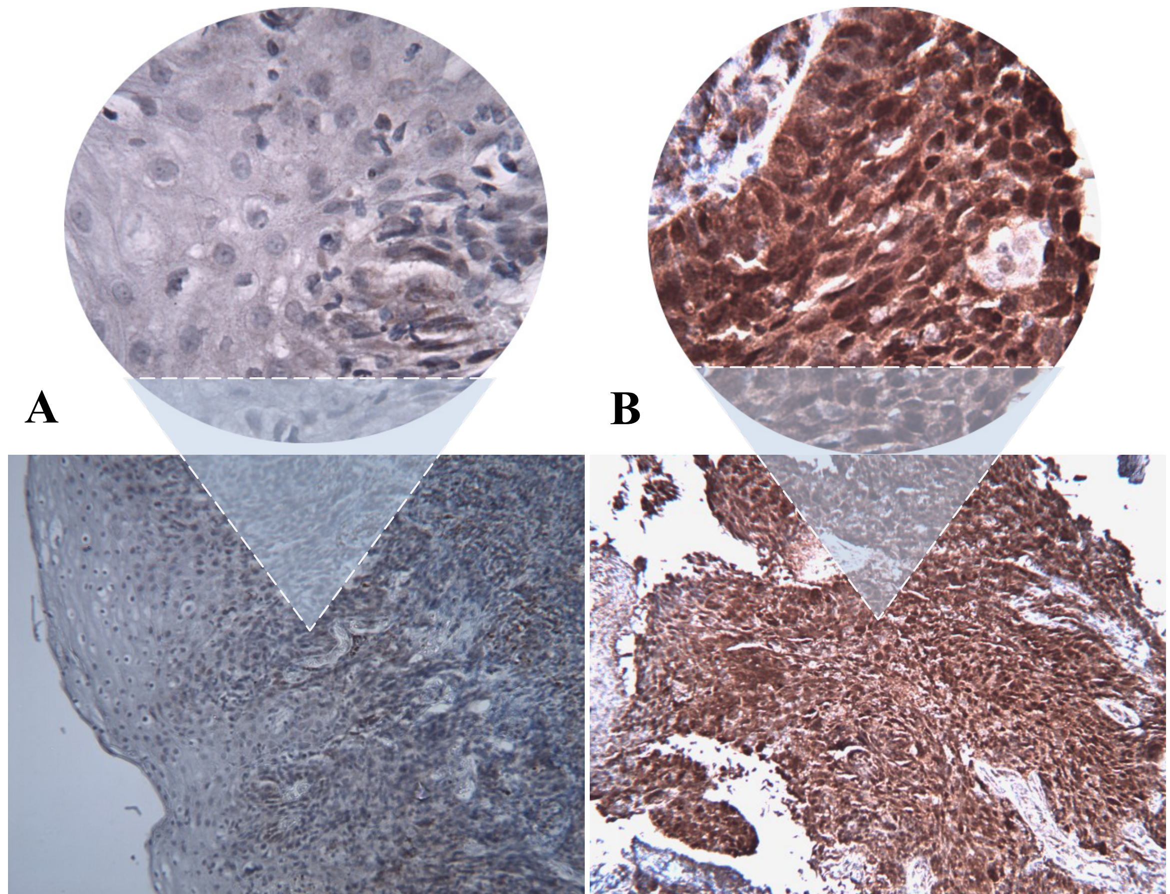



**Figure 3.23: Photomicrograph of p16 immunohistochemical staining.**
(A) Negative control, (B) p16 positive at x100 magnification (rectangular) and x400 magnification (circle)

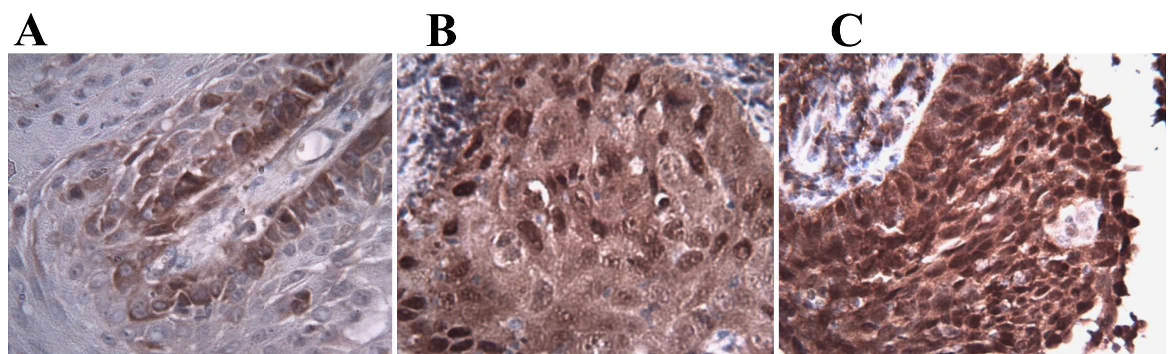



**Figure 3.24: Photomicrograph of p16 immunohistochemical staining intensity.**
(A) 1+, (B) 2+ and (C) 3+ at x400 magnification. 1+ score is the minimum for p16 positivity.

### 3.8.3 Correlation among demographic and clinical parameters associated with p16

A total of 47 of the 60 cases (78.3%) were positive for p16 immunohistochemistry based on consensus agreements among three independent blinded observers by semi-quantitative assessment (Figure 3.25). The demographic, risk factor and clinicopathological characteristics of oral cancer by p16 status are given in Table 3.29 and Table 3.30. Based on odds ratio, p16 status was significantly associated with an increase in relative risk compared to the reference group, male [3.5 (1.4 – 8.7), $p<0.01$], patients aged 66-97 [3.4 (1.5 – 8.0), $p<0.01$], tumour size $T_3$ [3.7 (1.2 – 11.3), $p<0.05$], dead [7.0 (0.1 – 1.7), $p<0.01$] and disease recurrence [3.5 (1.8 – 6.6), $p<0.01$] and regional lymph nodes, N2 [9.0 (1.1 – 71.0), $p<0.05$].

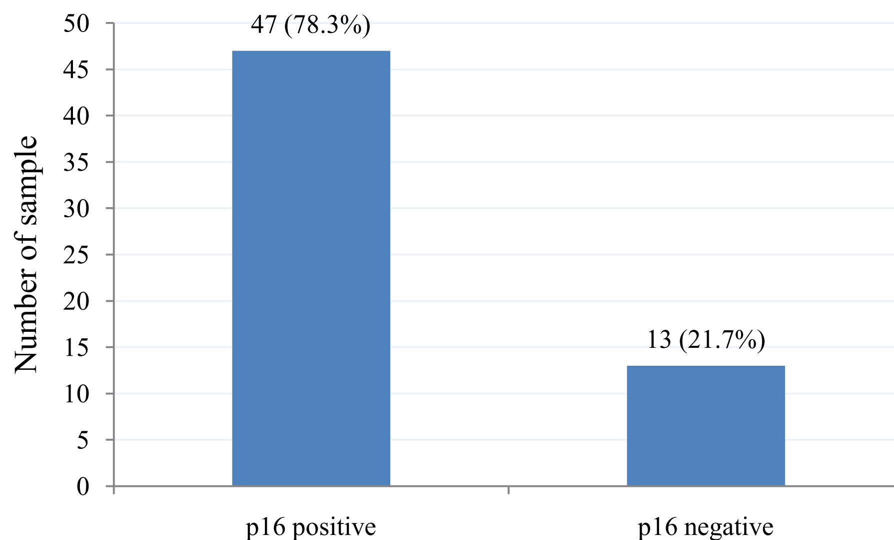



**Figure 3.25: The total score of p16 IHC in this pilot study.**

**Table 3.29: Demographic and risk factor characteristics of oral cancer by p16 status**

| Characteristics | *N* | p16-, *N*=13 (%) | P16+, *N*=47 (%) | Odds ratio (95% CI) | *p* value |
|---|---|---|---|---|---|
| Gender | | | | | |
| Male | 33 | 7 (53.8) | 26 (55.3) | 3.5 (1.4 – 8.7) | 0.007** |
| Female[α] | 27 | 6 (46.2) | 21 (44.7) | 1.0 | |
| Age group | | | | | |
| 36-65y[α] | 31 | 7 (53.8) | 24 (51.1) | 1.0 | 0.004** |
| 66-97y | 29 | 6 (46.2) | 23 (48.9) | 3.4 (1.5 – 8.0) | |
| Smoking | | | | | |
| No[α] | 20 | 6 (46.2) | 14 (29.8) | 1.0 | 0.082 |
| Yes | 40 | 7 (53.8) | 33 (70.2) | 2.3 (0.9 - 6.1) | |
| Alcohol | | | | | |
| No[α] | 10 | 2 (15.4) | 8 (17.0) | 1.0 | 0.080 |
| Yes | 50 | 11 (84.6) | 39 (83.0) | 4.0 (0.8 – 18.8) | |
| Smoking-Alcohol | | | | | |
| NSND[α] | 5 | 1 (7.7) | 4 (8.5) | 1.0 | 0.197 |
| SND | 5 | 1 (7.7) | 4 (8.5) | 4.0 (0.4 – 35.8) | |
| NSD | 15 | 5 (38.5) | 10 (21.3) | 4.0 (0.4 – 35.8) | |
| SD | 35 | 6 (46.2) | 29 (61.7) | 4.0 (0.7 – 5.9) | |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level; [α] - reference for odds ratio determination

**Table 3.30: Clinicopathologic characteristics of oral cancer by p16 status**

| Characteristics | *N* | p16-, *N*=13 (%) | p16+, *N*=47 (%) | Odds Ratio (95% CI) | *p* value |
|---|---|---|---|---|---|
| Anatomic site | | | | | |
| Buccal mucosa[α] | 1 | 0 (0) | 1 (2.1) | 1.0 | 0.179 |
| Floor of the mouth | 10 | 3 (23.1) | 7 (14.9) | - | |
| Alveolus | 4 | 2 (15.4) | 2 (4.3) | 2.3 (0.6 – 9.0) | |
| Soft palate | 3 | 0 (0) | 3 (6.4) | 1.0 (0.1 – 7.0) | |
| Pharynx | 1 | 0 (0) | 1 (2.1) | - | |
| Lower lip | 1 | 0 (0) | 1 (2.1) | - | |
| Retromolar pad | 9 | 3 (23.1) | 6 (12.8) | - | |
| Supraglottis | 1 | 0 (0) | 1 (2.1) | 2.0 (0.5 – 8.0) | |
| Tongue | 29 | 5 (38.5) | 24 (51.1) | - | |
| Tonsil | 1 | 0 (0) | 1 (2.1) | 4.8 (1.8 – 12.6) | |
| Tumour size | | | | | |
| Negative[α] | 2 | 0 (0) | 2 (4.3) | 1.0 | 0.027* |
| $T_1$ (<2 cm) | 24 | 6 (46.2) | 18 (38.3) | - | |
| $T_2$ (2-4 cm) | 19 | 4 (30.8) | 15 (31.9) | 3.0 (1.2 – 7.5) | |
| $T_3$ (>4 cm) | 1 | 0 (0) | 1 (2.1) | 3.7 (1.2 – 11.3) | |
| $T_4$ (>4 cm) | 14 | 3 (23.1) | 11 (23.4) | - | |
| Regional lymph nodes | 4 | 1 (7.7) | 3 (6.4) | 1.0 | 0.018* |
| Negative[α] | 28 | 8 (61.5) | 28 (46.7) | 3.0 (0.3 – 28.8) | |
| $N_0$ | 10 | 1 (7.7) | 10 (16.7) | 2.5 (1.1 – 5.7) | |
| $N_1$ | 18 | 3 (23.1) | 18 (30.0) | 9.0 (1.1 – 71.0) | |
| $N_2$ | | | | | |
| Distant metastasis | | | | | |
| Negative[α] | 4 | 1 (7.7) | 3 (6.4) | 1.0 | 0.341 |
| No distant metastasis | 56 | 12 (92.3) | 44 (93.6) | 3.0 (0.3 - 28.8) | |
| Tumour stage | | | | | |
| 0[α] | 6 | 1 (7.7) | 5 (10.6) | 1.0 | 0.310 |
| I | 17 | 6 (46.2) | 11 (23.4) | 5.0 (0.6 – 42.8) | |
| II | 4 | 0 (0) | 4 (8.5) | 1.8 (0.7 – 5.0) | |
| III | 7 | 2 (15.4) | 5 (10.6) | - | |
| IV | 26 | 4 (30.8) | 22 (46.8) | 2.5 (0.5 – 12.9) | |
| Disease outcome | | | | | |
| Alive | 24 | 3 (23.1) | 21 (44.7) | 7.0 (0.1 – 1.7) | 0.002** |
| Dead[α] | 36 | 10 (76.9) | 26 (55.3) | 1.0 | |
| Disease status | | | | | |
| No recurrence[α] | 54 | 12 (92.3) | 42 (89.4) | 1.0 | 0.000** |
| Recurrence | 6 | 1 (7.7) | 5 (10.6) | 3.5 (1.8 – 6.6) | |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level; [α] - reference for odds ratio determination

**Table 3.31: The correlations between demographic/clinical parameters and p16 status**

| Parameter 1 | Parameter 2 | Pearson Correlation | | Spearman's rho | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Correlation coefficient | Sig. (2-tailed) |
| **Within p16:** | | | | | |
| p16 status | p16 staining intensity | 0.769** | 0.000 | 0.756** | 0.000 |
| **Between p16 and demographic/clinical parameters:** | | | | | |
| p16 status | Gender | 0.012 | 0.926 | 0.012 | 0.926 |
| | Age group | 0.023 | 0.862 | 0.023 | 0.862 |
| | Disease outcome | -0.182 | 0.165 | -0.182 | 0.165 |
| | Anatomic site | 0.115 | 0.380 | 0.125 | 0.343 |
| | Tumour size | 0.007 | 0.956 | 0.012 | 0.925 |
| | Regional lymph nodes | 0.129 | 0.325 | 0.136 | 0.302 |
| | Distant metastasis | 0.022 | 0.870 | 0.022 | 0.870 |
| | Tumour stage | 0.120 | 0.359 | 0.126 | 0.337 |
| | Recurrence | 0.040 | 0.759 | 0.040 | 0.759 |
| | Smoking | 0.143 | 0.276 | 0.143 | 0.276 |
| | Alcohol | -0.018 | 0.891 | -0.018 | 0.891 |
| | Smoking-Alcohol | 0.057 | 0.664 | 0.099 | 0.452 |
| p16 staining intensity | Gender | 0.012 | 0.928 | 0.026 | 0.846 |
| | Age group | 0.049 | 0.712 | 0.042 | 0.751 |
| | Disease outcome | -0.258* | 0.047 | -0.258* | 0.046 |
| | Anatomic site | 0.131 | 0.319 | 0.103 | 0.434 |
| | Tumour size | 0.061 | 0.643 | 0.081 | 0.536 |
| | Regional lymph nodes | 0.085 | 0.516 | 0.090 | 0.495 |
| | Distant metastasis | 0.074 | 0.575 | 0.074 | 0.576 |
| | Tumour stage | 0.131 | 0.320 | 0.136 | 0.301 |
| | Recurrence | 0.039 | 0.764 | 0.017 | 0.897 |
| | Smoking | 0.237 | 0.068 | 0.255* | 0.049 |
| | Alcohol | -0.035 | 0.789 | -0.025 | 0.852 |
| | Smoking-Alcohol | 0.091 | 0.490 | 0.189 | 0.148 |

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlation analyses were carried out to search for any association existing between p16 positivity, p16 staining intensity and demographic and clinical parameters using Pearson's correlation and Spearman's correlation, rho in this cohort (Table 3.31). We found a very strong positive association between p16 positivity and p16 staining intensity. Unfortunately, neither the demographic nor clinical parameters were correlated with p16 status. A negative correlation was observed between p16 staining intensity with patient disease outcome ($r$ =-0.258, $p$<0.05) whereas positively associated with smoking ($rho$ = 0.255, $p$<0.05).

## 3.9 CORRELATION BETWEEN HPVs AND p16 EXPRESSION

When p16 expression is examined in association with HPV status, the symmetric measures table shows that Kappa varies from -0.046 to 0.070 (Table 3.32). Even though, there are positive Kappa values in three cases, but this is insufficient enough. For reliability measures, they should be high (≥0.70). The percentage of concordant based on positive kappa value ranging from 33% to as high as 55%. HR α-HPV/p16 and HPV16/p16 status association (shaded area) need to be focused on the future, we suggested that there was less specificity of IHC technique compared to PCR and HPV genotyping which affected the discordant values (darker shaded area). Therefore, at this point we concluded that none of the HPV and p16 status demonstrated remarkably convincing agreements and consistency. Statistical significance is not relevant for reliability measures.

**Table 3.32: Cross-tabulation of HPV versus p16 status**

| HPV expression | *N* | p16 expression p16 - (%) | p16 expression p16 + (%) | Concordance[a] (%) | κ | *p* value |
|---|---|---|---|---|---|---|
| Overall HPV (-) | 20 | 4 (6.7) | 16 (26.7) | 56.7 | -0.027 | 0.825 |
| Overall HPV(+) | 40 | 9 (15.0) | 31 (51.7) | | | |
| α-HPV (-) | 26 | 6 (10.0) | 20 (33.3) | 55.0 | 0.026 | 0.817 |
| α-HPV (+) | 34 | 7 (11.7) | 27 (45.0) | | | |
| HR α-HPV (-) | 49 | 12 (20.0) | 37 (61.7) | 36.7 | 0.068 | 0.263 |
| HR α-HPV (+) | 11 | 1 (1.7) | 10 (16.7) | | | |
| HPV16 (-) | 53 | 13 (21.7) | 40 (66.7) | 33.3 | 0.070 | 0.139 |
| HPV16 (+) | 7 | 0 (0) | 7 (11.7) | | | |
| LR α-HPV (-) | 26 | 5 (8.3) | 21 (35.0) | 51.7 | -0.046 | 0.689 |
| LR α-HPV (+) | 34 | 8 (13.3) | 26 (43.3) | | | |
| β-HPV (-) | 49 | 10 (16.7) | 39 (65.0) | 30.0 | -0.030 | 0.617 |
| β-HPV (+) | 11 | 3 (5.0) | 8 (13.3) | | | |

[a](Concordance positive and concordance negative)x100/total specimens

**Table 3.33: Chi-square analysis of prevalence of p16 positivity with HPV status**

| HPV status | $N$ | p16 status: p16 - (N = 13) (%) | p16 + (N = 47) (%) | $\chi^2$ | $p$ value |
|---|---|---|---|---|---|
| Overall HPV (-) | 20 | 4 (20.0) | 16 (80.0) | 0.049 | 0.825 |
| Overall HPV(+) | 40 | 9 (22.5) | 31 (77.5) | | |
| α-HPV (-) | 26 | 6 (23.1) | 20 (76.9) | 0.054 | 0.817 |
| α-HPV (+) | 34 | 7 (20.6) | 27 (79.4) | | |
| HR α-HPV (-) | 49 | 12 (24.5) | 37 (75.5) | 1.255 | 0.263 |
| HR α-HPV (+) | 11 | 1 (9.1) | 10 (90.9) | | |
| HPV16 (-) | 53 | 13 (24.5) | 40 (75.5) | 2.192 | 0.139 |
| HPV16 (+) | 7 | 0 (0) | 7 (100.0) | | |
| LR α-HPV (-) | 26 | 5 (19.2) | 21 (80.8) | 0.160 | 0.689 |
| LR α-HPV (+) | 34 | 8 (23.5) | 26 (76.5) | | |
| β-HPV (-) | 49 | 10 (20.4) | 39 (79.6) | 0.249 | 0.617 |
| β-HPV (+) | 11 | 3 (27.7) | 8 (72.7) | | |

**Table 3.34: Chi-square analysis of prevalence of p16 staining intensity with HPV status**

| HPV status | $N$ | p16 staining intensity: Negative (%) | 1+ (%) | 2+ (%) | 3+ (%) | $\chi^2$ | $p$ value |
|---|---|---|---|---|---|---|---|
| Overall HPV (-) | 20 | 4 (20.0) | 9 (45.0) | 7 (35.0) | 0 (0) | 1.886 | 0.596 |
| Overall HPV(+) | 40 | 9 (22.5) | 14 (35.0) | 14 (35.0) | 3 (7.5) | | |
| α-HPV (-) | 26 | 6 (23.1) | 10 (38.5) | 10 (38.5) | 0 (0) | 2.494 | 0.476 |
| α-HPV (+) | 34 | 7 (20.6) | 13 (38.2) | 11 (32.4) | 3 (8.8) | | |
| HR α-HPV (-) | 49 | 12 (24.5) | 19 (38.8) | 18 (36.7) | 0 (0) | 14.59** | 0.002 |
| HR α-HPV (+) | 11 | 1 (9.1) | 4 (36.4) | 3 (27.3) | 3 (27.3) | | |
| HPV16 (-) | 53 | 13 (24.5) | 21 (39.6) | 19 (35.8) | 0 (0) | 24.722** | 0.000 |
| HPV16 (+) | 7 | 0 (0) | 2 (28.6) | 2 (28.6) | 3 (42.9) | | |
| LR α-HPV (-) | 26 | 5 (19.2) | 11 (42.3) | 9 (34.6) | 1 (3.8) | 0.439 | 0.932 |
| LR α-HPV (+) | 34 | 8 (23.5) | 12 (35.3) | 12 (35.3) | 2 (5.9) | | |
| β-HPV (-) | 49 | 10 (20.4) | 19 (38.8) | 18 (36.7) | 2 (4.1) | 0.890 | 0.828 |
| β-HPV (+) | 11 | 3 (27.3) | 4 (36.4) | 3 (27.3) | 1 (5.0) | | |

**. Correlation is significant at the 0.01 level (2-tailed).

Correlation analyses were performed seeking any existing association between HPV status and p16 positivity (Table 3.33) and p16 staining intensity by Pearson Chi-square correlation (Table 3.34). We found that no significant association existed between HPV and p16 status. In other words, HPV status was not significantly different whether the status of p16 was positive or negative. Positive association was observed between p16 staining intensity with HR-HPV status ($\chi^2$ = 14.59, *df* = 3, *N* = 60, $p$<0.001) and HPV16 status ($\chi^2$ = 24.72, *df* = 3, *N* = 60, $p$<0.001). Therefore, we concluded that HR-HPV and HPV16 status were significantly associated with p16 staining intensity.

## 3.10 SURVIVAL ANALYSIS

Survival was measured in months from the date of diagnosis until death or until the patient was last known to be alive. The Kaplan-Meier curve of survival followed by Log Rank (Mantel-Cox) test was used to investigate whether there was an association between the clinical parameters and patients survival. All survival curves were generated in SPSS ver.16. Continuous data were divided by the median and the comparison was made using the log-rank test. The Kaplan-Meier is the univariate version of survival analysis. Then, by performing Cox regression analysis it will also assist the assessment of the actual impact of confounders on the survival of the patient.

Cox proportional hazard models were used to assess the relationships between p16 and HPV and to adjust for factors previously found to be prognostically significant in oral cancer. These analyses included age, alcohol, smoking, tumour stage, tumour site also were examined in separate models. All variables were assessed for the proportional

hazard assumption before inclusion in the models. HPV and p16 status were examined separately and then four groups: HPV/p16+ (reference group), HPV/p16-negative, HPV-negative/p16+, HPV-negative/p16-negative. The magnitude of the associations was assessed with hazard ratios (HRs) calculated from the Cox regression models. Statistically significance was based on two-tailed tests and p-values $\leq$ 0.05. Statistical analyses were performed using SPSS 16.0.

### 3.10.1 Kaplan-Meier Survival Analysis

The results suggested that the overall patient survival was worst in six groups (Table 3.35):

1. Pharynx, lower lip, tonsil and buccal mucosa compared with other anatomic sites ($p<0.01$) (Figure 3.26).
2. Tumour size, T3 compared with T1 ($p<0.01$) (Figure 3.27).
3. Tumour stage II compared with stage 0 ($p<0.01$) (Figure 3.28).
4. Non-drinkers compared to drinkers, ($p<0.01$) (Figure 3.29).
5. Non-smokers and non-drinkers compared to non-smokers but drinkers, ($p<0.01$) (Figure 3.30).
6. Patients with positive β-HPV type compared to the negative one, ($p<0.01$) (Figure 3.31).

**Table 3.35: The prognostic significance from overall survival analysis**

| Parameter ($N$=60) | Log Rank (Mantel-Cox) | | |
|---|---|---|---|
| | *Chi-Square* | *df* | *Sig.* |
| Gender | 0.587 | 1 | 0.444 |
| Age group | 2.887 | 1 | 0.089 |
| Anatomic site | 33.893 | 9 | 0.000** |
| Tumour size | 15.845 | 4 | 0.003** |
| Regional Lymph Nodes | 4.150 | 3 | 0.246 |
| Distant Metastasis | 0.512 | 1 | 0.474 |
| Tumour stage | 31.566 | 4 | 0.000** |
| Recurrence | 0.267 | 1 | 0.605 |
| Smoking | 1.408 | 1 | 0.235 |
| Alcohol | 16.128 | 1 | 0.000** |
| Smoking-Alcohol | 31.205 | 3 | 0.000** |
| Overall HPV status | 1.373 | 1 | 0.241 |
| High risk HPV status | 0.658 | 1 | 0.417 |
| HPV16 status | 0.201 | 1 | 0.654 |
| Low risk HPV status | 0.021 | 1 | 0.885 |
| α-HPV status | 0.222 | 1 | 0.637 |
| β-HPV status | 10.695 | 1 | 0.001** |
| HPV's mode of infection | 2.861 | 3 | 0.414 |
| p16 status | 2.293 | 1 | 0.130 |
| HPV/p16 status | 3.912 | 3 | 0.271 |
| High risk HPV/p16 status | 3.448 | 3 | 0.328 |
| HPV16/p16 status | 2.816 | 3 | 0.245 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

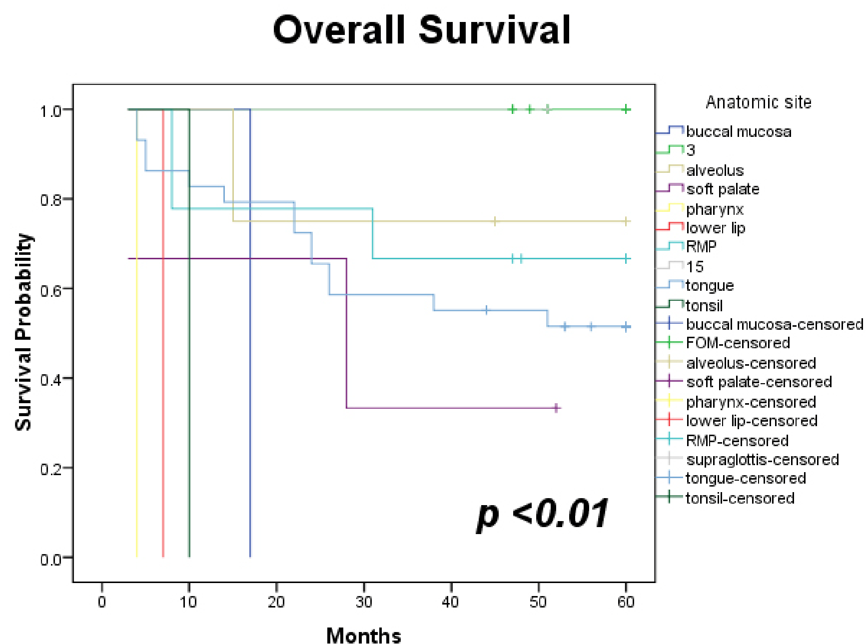



**Figure 3.26: An association amongst anatomic sites and overall survival as generated by Kaplan-Meier test.** Vertical tick marks on curves indicate censored observations.

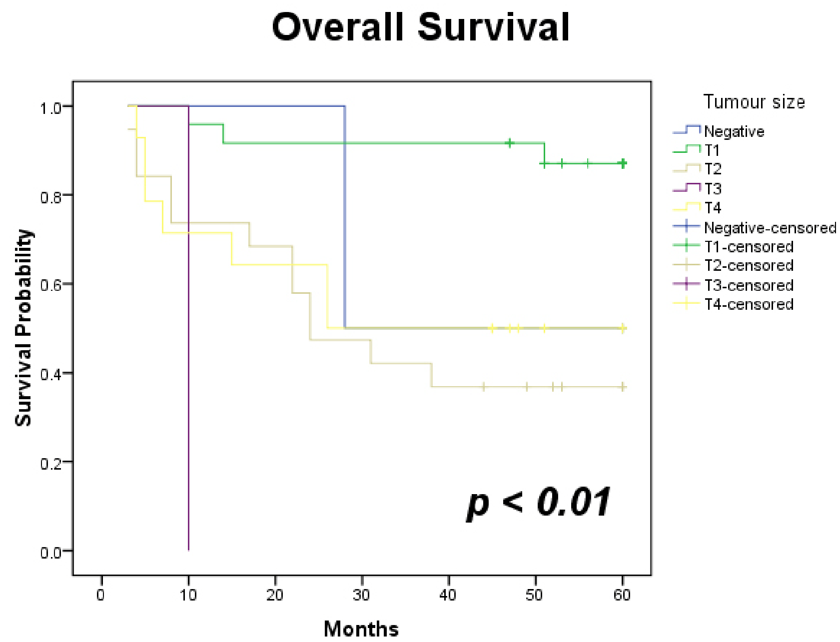



**Figure 3.27: An association amongst tumour sizes and overall survival as generated by Kaplan-Meier test.**

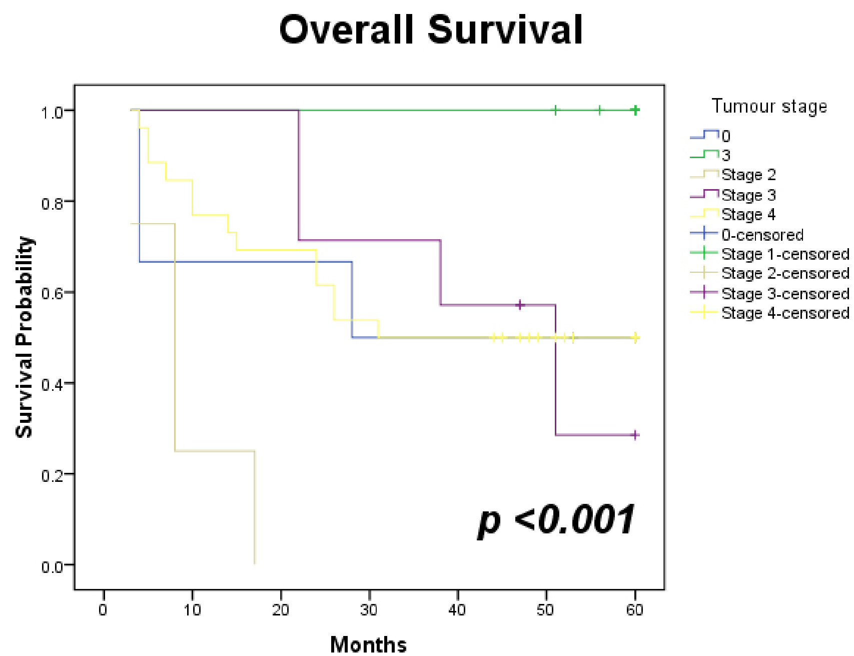



**Figure 3.28: An association amongst tumour stage and overall survival as generated by Kaplan-Meier test.**

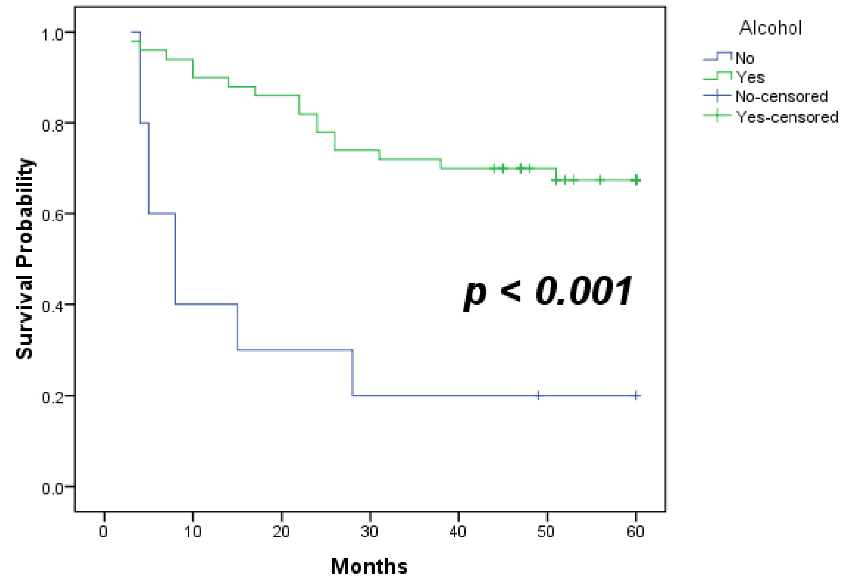



**Figure 3.29: An association between patients with alcohol exposure and overall survival as generated by Kaplan-Meier test.**

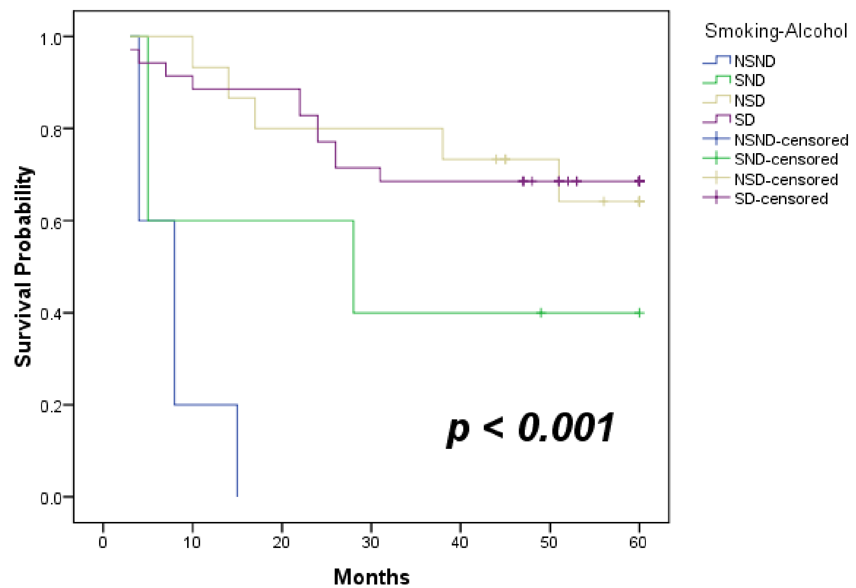



**Figure 3.30: An association amongst smoking-alcohol exposure and overall survival as generated by Kaplan-Meier test.**

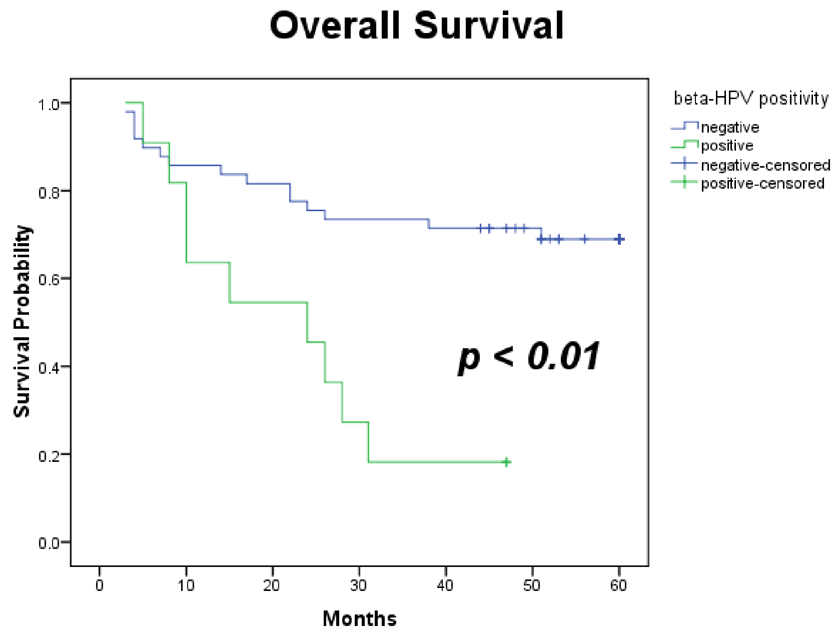



**Figure 3.31: An association between patients with positive β-HPV types and overall survival as generated by Kaplan-Meier test.**

### 3.10.2 Survival analysis according to the Cox Regression Model

Multifactorial effect on patient survival as analysed by Cox regression model. These prognostic factors exhibited independently an agreement with overall survival analysis by Kaplan-Meier test except for anatomic site and tumour stage (Table 3.36).

**Table 3.36: Patient survival analysis by Cox regression model**

| Parameter (*N*=60) | Cox Regression Model | | |
|---|---|---|---|
| | *Wald* | *df* | *Sig.* |
| Gender | 0.573 | 1 | 0.449 |
| Age group | 2.733 | 1 | 0.098 |
| Anatomic site | 13.242 | 9 | 0.152 |
| Tumour size | 11.494 | 4 | 0.022* |
| Regional Lymph Nodes | 3.847 | 3 | 0.278 |
| Distant Metastasis | 0.484 | 1 | 0.487 |
| Tumour stage | 9.364 | 4 | 0.053 |
| Recurrence | 0.260 | 1 | 0.610 |
| Smoking | 1.360 | 1 | 0.243 |
| Alcohol | 13.039 | 1 | 0.000** |
| Smoking-Alcohol | 19.478 | 3 | 0.000** |
| Overall HPVstatus | 1.320 | 1 | 0.251 |
| High risk HPV status | 0.635 | 1 | 0.425 |
| HPV16 status | 0.103 | 1 | 0.748 |
| Low risk HPVstatus | 0.021 | 1 | 0.885 |
| α-HPV status | 0.218 | 1 | 0.64 |
| β-HPVstatus | 9.175 | 1 | 0.002** |
| HPV's mode of infection | 2.694 | 3 | 0.441 |
| p16 status | 2.114 | 1 | 0.146 |
| HPV/p16 status | 1.459 | 3 | 0.692 |
| High risk HPV/p16 status | 2.661 | 3 | 0.447 |
| HPV16/p16 status | 2.598 | 3 | 0.273 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

## 3.11 CONCLUSIONS

Our results thus far from a small cohort in this pilot study could conclude:

### 1.A Optimisation of DNA extraction

- For samples in the form of cell suspension, sample duplication and repeated measurements for DNA quality and quantity was not necessary since no significance different observed within and between cells ($p>0.05$).
- Even though, statistically no significant qualitative and quantitative variations observed within FFPETs by repeated readings ($p>0.05$) variations existed between samples ($p<0.05$). Therefore, it was reliable and a good practice having each

sample in duplicate and by taking an average of the measurements for each sample for DNA quality and quantity comparison.

- Based on overall performance comparison, Qiagen kits were selected for future DNA extraction method used and 5 coupes of 5 μm paraffin embedded tissues was the best option for the DNA purity and DNA yield.

**1.B Optimisation of Polymerase Chain Reaction**

- 0.01 ng of DNA template was the minimum quantity required to be detected in 2% agarose gel.
- 2.5 mM $MgCl_2$ concentration (commercial premix) produced relatively more specific amplicon than 3.0 mM $MgCl_2$ (manually prepared). Therefore, the former will be utilised for future amplification.
- $3^5$ dilution (or 243x dilution) of the initial 450 bp amplicon as a DNA template gave the highest specificity for the nested or semi-nested amplicons, 140bp or 190 bp.
- The band intensity of the 140 bp amplicons relatively doubled after reamplification (without removing them from the thermocycler) and was free from other non-specific PCR products and this would be suggested for future amplification for nested PCR.

**2. HPV prevalence in oral squamous cell carcinoma samples**

- 40/60 (66.7%) overall HPV positivity was observed in this cohort.
- It was postulated that more than single mode of HPV infections existed in the same sample. But this needs to be elucidated in a more advanced future study such as utilising viral load and laser capture microdissection (LCM) approaches.

- Overall, the highest proportion of LR-HPV observed particularly, HPV6 (34/56 or 60.7%) followed by an equal percentage of HR-HPVs and β-HPVs, 11/56 (19.6%)
- The actual roles played by β-HPV (commonly found in cutaneous) in oral mucosal specimen remains a mystery.
- Two α-HPV untypable (both were present in postulated multiple HPV infection) and three β-HPV untypable (two were detected in postulated single HPV infection and one as multiple HPV infection with α-HPV types) could be the novel HPV types. Since conventional PCR, PM-PCR RHA and $SFP_{10}$-$LiPA_{25}$ (version 1) approaches failed to identify them, other methods should be employed as a follow up for further HPV genotyping purposes.
- Subsites analysis was unreliable due to very low sample size in certain groups.
- HPV positivity was associated with other demographic and clinical risk factors such as smoking and patient disease outcome each with ($p<0.05$).
- No direct association observed between HR-HPV or HPV16 with patient disease outcome.

**3A. p16 positivity in oral squamous cell carcinoma samples**

- 47/60 (78.3%) p16 positivity observed in this cohort
- p16 status was significantly associated with an increased relative risk in male, older patients, tumour size, $T_3$ , patient with disease recurrence and having positive lymph nodes.
- A positive correlation was observed between p16 staining intensity with p16 status and smoking, but negatively associated with patient disease outcome.

### 3B. Association between HPVs and p16

- Chi-square analysis demonstrated that p16 staining intensity is associated with HR-HPV and HPV16 status. Nevertheless, no association was observed for overall HPV types and p16 status.
- No direct association between HPVs and p16 expression was observed and the main cause was due to the lack of power in analysis.

### 3C. Prognostic value of HPVs and p16

- The overall patients survival were worst in four parameters; tumour size T3, non-drinkers, NSND group and patients positive with β-HPV type. These were satisfied by Cox regression analysis.

# CHAPTER 4

# HPVs AND ORAL DISEASE PROGRESSION

## 4.1 OVERVIEWS

Overviews of aims of this chapter are given as follows:

- To extract DNA from 183 FFPETs which was composed of benign (*n*=84), dysplasia (*n*=12), squamous cell carcinoma (*n*=80) and normal tissues (*n*=7)
- PCR amplification for L1 HPV detection
- Purification and DNA sequencing of the PCR products
- To establish HPV prevalence, demographic and clinicopathological parameters in benign, dysplasia and carcinoma.
- To determine whether there is an association with disease progression
- Potential association with survival for HPV (for carcinoma group only)

## 4.2 CLINICAL DATA

A total of 183 cases were used with histologically confirmed benign, dysplasia and squamous cell carcinoma and normal tissues of various anatomic sites evaluated at the Unit of Oral Surgery and Medicine, University of Dundee, between 2005 and 2009. The male:female ratio was 56:44 with the mean and standard deviation (SD) age of 57.4 $\pm$ 15.4 years, ranging from 16-99 years. An average age for male was 58.8 $\pm$ 16.9 and 56.2 $\pm$ 14.0 for female. As to the tumour loci, 46 cases (25.1%) were on buccal mucosa; tongue, 44 cases (24%); floor of the mouth, 23 cases (12.6%); retromolar pad, 5 cases

(2.7%); gingivae, 10 cases (5.5%); hard palate, 3 cases (1.6%); soft palate, 39 cases (21.3%); upper lip, 2 cases (1.1%), lower lip, 9 cases (4.9%) and one case (0.5%) for skin and tonsil. The clinico-pathological parameters recorded in each tumour consist of the primary tumour (T), regional lymph node (N). The clinical details of 176 samples from those patients of three pathological groups i.e. benign (B), dysplasia (D) and carcinoma (C) [Appendix 30 (A)] and are summarized in Table 4.1. The overall median (age 57) was used to classify those patients into two age groups. The histopathological details of patients with carcinoma were considered in Table 4.2. Another seven samples of normal tissues (N) were randomly selected. The clinical details are shown in Appendix 30 (B).

## 4.3 DNA EXTRACTION FROM ORAL DISEASE PROGRESSION STUDY

The raw data for qualitative and quantitative measurements of extracted DNA from normal tissues [Appendix 30 (C)] and three pathological groups is given in Appendix 31 (benign), Appendix 32 (dysplasia) and Appendix 33 (carcinoma). The overall purity and yield of extracted DNA from FFPETs is given in Appendix 54.

## 4.4 β-GLOBIN DETECTION BY PCR FOR DNA QUALITY DETERMINATION

PCR amplication was undertaken in triplicate for each diluted DNA from 2.1.21 according to the procedure mentioned in 2.1.22, Materials and Methods. The first attempt successfully amplified this fragment in 168 out of 183 samples (91.8%). Finally, all 183 samples (100%) were positive for β-globin gene and suitable as a

template DNA for L1 gene detection of HPV. The PCR results for β-globin detection were given separately in normal tissues [Appendix 30 (D)] and each pathological group: Appendix 34 (benign), Appendix 35 (dysplasia) and Appendix 36 (carcinoma).

**Table 4.1: Clinical details of patients in each pathological group**

| Characteristic | *N* | Benign *N* (%) | Dysplasia *N* (%) | Carcinoma *N* (%) |
|---|---|---|---|---|
| Total | 176 | 84 (47.7) | 12 (6.8) | 80 (45.5) |
| Gender | | | | |
| Female | 99 | 45 (45.5) | 5 (5.0) | 49 (49.5) |
| Male | 77 | 39 (50.6) | 7 (9.1) | 31 (40.3) |
| Age group$^{\alpha}$ (range, 16-99y) | | | | |
| 16-57y | 92 | 52 (56.5) | 2 (2.2) | 38 (41.3) |
| 58-99y | 84 | 32 (38.1) | 10 (11.9) | 42 (50.0) |
| Anatomic site | | | | |
| Buccal mucosa | 45 | 41 (91.1) | 1 (2.2) | 3 (6.7) |
| Floor of the mouth | 23 | 3 (13.0) | 8 (34.8) | 12 (52.2) |
| Gingivae | 10 | 6 (60.0) | 0 (0.0) | 4 (40.0) |
| Hard palate | 3 | 2 (66.7) | 1 (33.3) | 0 (0.0) |
| Soft palate | 39 | 3 (7.7) | 2 (5.1) | 34 (87.2) |
| Upper lip | 2 | 2 (100.0) | 0 (0.0) | 0 (0.0) |
| Lower lip | 8 | 4 (50.0) | 0 (0.0) | 4 (50.0) |
| Retromolar pad | 4 | 1 (25.0) | 0 (0.0) | 3 (75.0) |
| Skin | 1 | 1 (100.0) | 0 (0.0) | 0 (0.0) |
| Tongue | 41 | 21 (51.2) | 0 (0.0) | 20 (48.8) |
| Disease status | | | | |
| No recurrence | 171 | 84 (49.1) | 12 (7.0) | 75 (43.9) |
| Recurrence | 5 | 0 (0.0) | 0 (0.0) | 5 (100.0) |
| Disease outcome (*N*=80) | | | | |
| Alive | 45 | 0 (0.0) | 0 (0.0) | 45 (100.0) |
| Dead | 35 | 0 (0.0) | 0 (0.0) | 35 (100.0) |

*Note: N, number of patients,* $^{\alpha}$ *- Age group was based on median age*

**Table 4.2: Histopathological details of patients with carcinoma**

| Characteristic | *N* | % |
|---|---|---|
| Total | 80 | 100 |
| Tumour size | | |
| $T_1$ (<2 cm) | 41 | 51.2 |
| $T_2$ (2-4 cm) | 22 | 27.5 |
| $T_3$ (>4 cm) | 13 | 16.2 |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | 5.0 |
| Regional Lymph Nodes | | |
| $N_0$ | 67 | 83.8 |
| $N_1$ | 7 | 8.8 |
| $N_2$ | 6 | 7.5 |
| Histological grade | | |
| Well differentiated | 9 | 11.2 |
| Moderately differentiated | 58 | 72.5 |
| Poorly differentiated | 11 | 13.8 |
| Basaloid | 2 | 2.5 |

*Note: N, number of patients*

## 4.5 SUMMARY OF THE PCR AMPLIFICATION OF L1 HPV GENE DETECTIONS

Similarly, the details of PCR results for each sample of L1 HPV gene detection were given in Appendices 34-36. Figure 4.1 illustrates the overall PCR results for L1 HPV detection according to the strategies mentioned in appropriate section in Materials and Methods. The overall potentially positive PCR amplification for L1 HPV gene is shown in Table 4.3. The actual positive PCR for L1 HPV will be determined later via automated DNA sequencing and DNA sequences alignment results. The PCR positivity based on three pathological groups is given in Table 4.4.

**Table 4.3: Overall PCR results for L1 HPV gene detection**

| Type of PCR amplification | Expected amplicon sizes (%) | | |
|---|---|---|---|
| | 450 bp | 190 bp | 140 bp |
| Independent PCR (positive) | 2 (1.1) | | |
| Independent PCR (negative) | 174 (98.9) | | |
| Total samples tested | 176 | | |
| Semi-nested PCR (positive) | $2^{\alpha}$ | 2 (1.1) | |
| Semi-nested PCR (positive) | $174^{\beta}$ | 124 (70.5) | |
| Semi-nested PCR (negative) | | 50 (28.4) | |
| Total samples tested | | 176 | |
| Nested PCR (positive) | | $2^{\alpha}$ | 2 (1.1) |
| Nested PCR (positive) | | $124^{\beta}$ | 117 (66.5) |
| Nested PCR (false positive) | | | 7 (4.0) |
| Nested PCR (positive) | | $50^{\beta}$ | 18 (10.2) |
| Nested PCR (negative) | | | 32 (18.2) |
| | | | 176 |
| Presumptive PCR positive for L1 HPV | 2 (1.1) | 126 (71.6) | 137 (77.8) |
| PCR negative for L1 HPV | 174 (98.9) | 50 (28.4) | 39 (22.2) |
| Total sample tested | 176 | 176 | 176 |

Note:
$^{\alpha}$ - First round PCR which was positive as the template for either semi-nested or nested PCR amplifications
$^{\beta}$ - First round PCR which was negative as the template for either semi-nested or nested PCR amplifications

**Table 4.4: Summary of PCR results of L1 HPV gene detection (pathological groups)**

| Type of PCR amplification | Number of samples (%) | | | Total |
|---|---|---|---|---|
| | Benign | Dysplasia | Carcinoma | |
| Independent PCR | 0 (0.0) | 1 (8.3) | 1 (12.5) | 2 (1.1) |
| Semi-nested PCR (Positive) | 61 (72.6) | 9 (75.0) | 56 (70.0) | 126 (71.6) |
| Nested PCR (false positive) | 4 (4.8) | 0 (0.0) | 3 (37.5) | 7 (4.0) |
| Nested PCR (Positive) | 61 (72.6) | 9 (75.0) | 67 (83.8) | 137 (77.8) |
| Presumptive PCR positive for L1 HPV | 61 (72.6) | 9 (75.0) | 67 (83.8) | 137 (77.8) |
| PCR negative for L1 HPV | 23 (27.4) | 3 (25.0) | 13 (16.2) | 39 (22.2) |
| Total sample tested | 84 | 12 | 80 | **176** |

## 4.6 MULTIPLE DNA SEQUENCES ALIGNMENT VIA BLASTn

In total of three pathological groups, 90 out of 137 amplicons (65.7%) were positively identified as HPVs which demonstrated very high expectation value (*E*) of the alignment (Mount, 2001) (Appendix 37-39). In total, forty seven amplicons (34.3%) were considered negative for HPVs via multiple DNA sequence alignment. Generally, a very low degree of DNA sequence similarity was observed in those sequences and less than 10% identity involving 10 base nucleotides or less (data not shown). Those amplicons (which were initially positive in PCR for L1 HPV detection) were included in DNA sequencing to ensure that we did not exclude any possibility of HPV positivity in the samples tested. Therefore, we concluded that 86 of 176 samples (48.9%) were negative for HPVs of which 39/86 (45.3%) and 47/86 (54.7%) were identified via PCR and DNA sequencing, respectively. In normal tissues, one of five amplicons (20%) was positively identified as HPV. We concluded that six out of seven normal tissue samples (85.7%) were negative for HPVs of which 2/6 (33.3%) and 4/6 (66.7%) were identified via PCR and DNA sequencing respectively.

## 4.7 A COMPARISON OF HPV DNA EXPRESSION IN DIFFERENT SAMPLES

We found that HPV16 was present in one of seven normal tissues (14.3%). It was previously reported elsewhere that HPV positivity in normal tissues varied from as low as 0% to 100%. Table 4.5 and Figure 4.1 show the positively detected HPV types, 43/84 (51.2%) in benign, 5/12 (41.7%) in dysplasia and 42/80 (52.5%) in carcinoma samples tested. Overall, we found that 54/90 (60.0%) positive for LR α-HPV (only HPV6

detected), 26/90 (28.9%) for HR α-HPV and 10/90 (11.1%) for other HPV types. HR α-HPV types were positively detected, 12/84 (14%) in benign, 13/80 (16%) carcinoma but 1/12 (8%) was present in dysplasia samples (Figure 4.2). Across the pathological group, HPV6 was the most predominantly observed compared to other HPV types, 28/43 (65.1%) in benign, 2/5 (40%) in dysplasia and 24/42 (57%) in carcinoma. Within HR α-HPV, the ratio of HPV16:HPV18 was 9:4. The proportion of HPV16 observed across pathological group was 7/43 (16.3%) in benign, 1/5 (20%) in dysplasia and 10/42 (23.8%) in carcinoma. The comparison of HPV prevalence in normal tissues and three pathological groups was not statistically significant. Therefore, HPV prevalence comparison was carried out among three pathological groups.

Figure 4.2 and Figure 4.3 show the distribution and the breakdown of each HPV type detected in the three pathological groups. HPV16 was predominantly detected within HR α-HPV types in all pathological groups, 7/84 (8.3%) in benign and 10/80 (12.5%) in carcinoma and 1/12 (8.3%) present in dysplasia samples (Table 4.5). The ratio of total LR α-HPV:HR α-HPV type was approximately 2:1 observed across the pathological groups. In addition, only single HPV type, HPV6 observed in LR α-HPV group. HPV33 and HPV113 were found only in benign tumours whereas HPV64, HPV103 and HPV109 were detected only in the carcinoma group. HPV100, on the other hand, was present in all pathological groups. In the benign group, 23/84 (27.4%) true negatives for HPV detection were observed at the pre-DNA sequencing stage and false positive results of 18/84 (21.4%) at the post-DNA sequencing stage. Similarly, it was noted that false positive PCR, 4/12 (33.3%) and true negative PCR, 3/12 (25.0%) results were almost comparable in the dysplasia group. Nevertheless, it seems that both

negative results were quite significantly different in the carcinoma group, 13/80 (16.3%) and 25/80 (31.3%) for true negative and false positive PCR, respectively.

**Table 4.5: Summary of HPV types detected in each pathological group**

| HPV types detected | Number of samples HPV positive (%) | | | Total |
|---|---|---|---|---|
| | Benign | Dysplasia | Carcinoma | |
| LR α-HPV | | | | |
| HPV6 | 28 (100.0) | 2 (100.0) | 24 (100.0) | 54 |
| Total | 28 | 2 | 24 | 54 (60.0) |
| HR α-HPV | | | | |
| HPV16 | 7 (61.5) | 1 (100.0) | 10 (76.9) | 18 |
| HPV18 | 5 (38.5) | - | 3 (23.1) | 8 |
| Total | 12 | 1 | 13 | 26 (28.9) |
| Other HPV types | | | | |
| HPV33 | 1 (33.3) | - | - | 1 |
| HPV64 | - | - | 1 (20.0) | 1 |
| HPV100 | 1 (33.3) | 2 (100.0) | 2 (40.0) | 5 |
| HPV103 | - | - | 1 (20.0) | 1 |
| HPV109 | - | - | 1 (20.0) | 1 |
| HPV113 | 1 (33.3) | - | - | 1 |
| Total | 3 | 2 | 5 | 10 (11.1) |
| Presumptive PCR positive for L1 HPV | 61 (72.6) | 9 (75.0) | 67 (83.8) | 137 |
| True positive PCR for L1 HPV | **43 (51.2)** | **5 (41.7)** | **42 (52.5)** | **90** |
| False positive PCR for L1 HPV | 18 (21.4) | 4 (33.3) | 25 (31.25) | 47 |
| True negative PCR for L1 HPV | 23 (27.4) | 3 (25.0) | 13 (16.25) | 39 |
| Total sample tested | 84 | 12 | 80 | **176** |

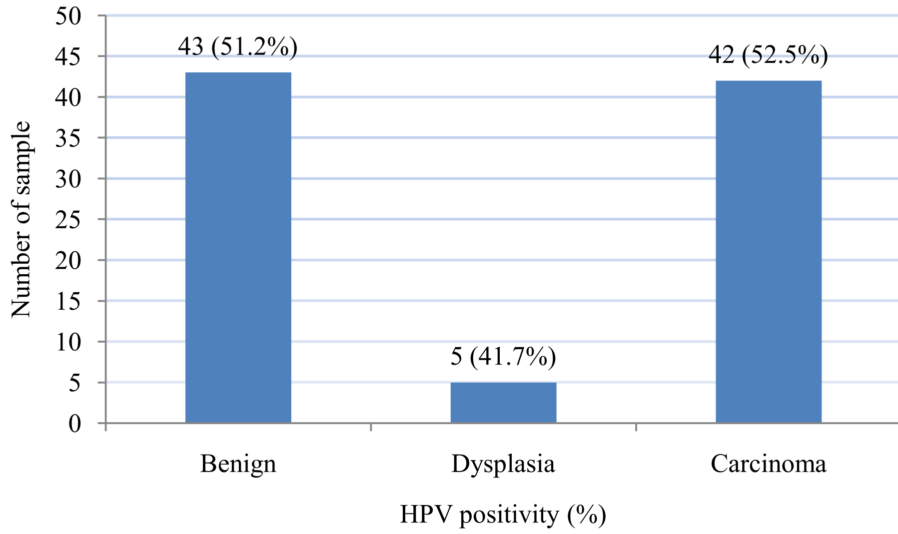



**Figure 4.1: HPV types positively detected in each pathological group**

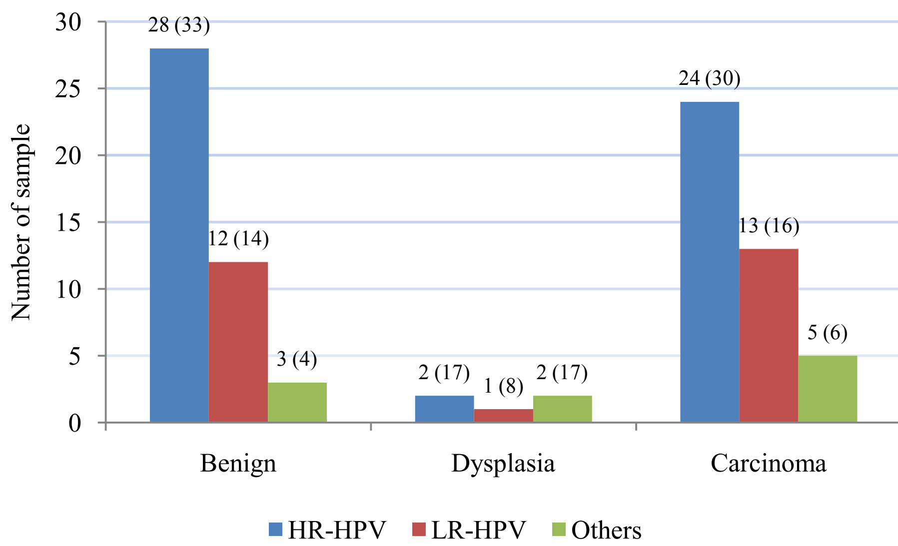



**Figure 4.2: HPV types distribution in each pathological group, *N* (%).**

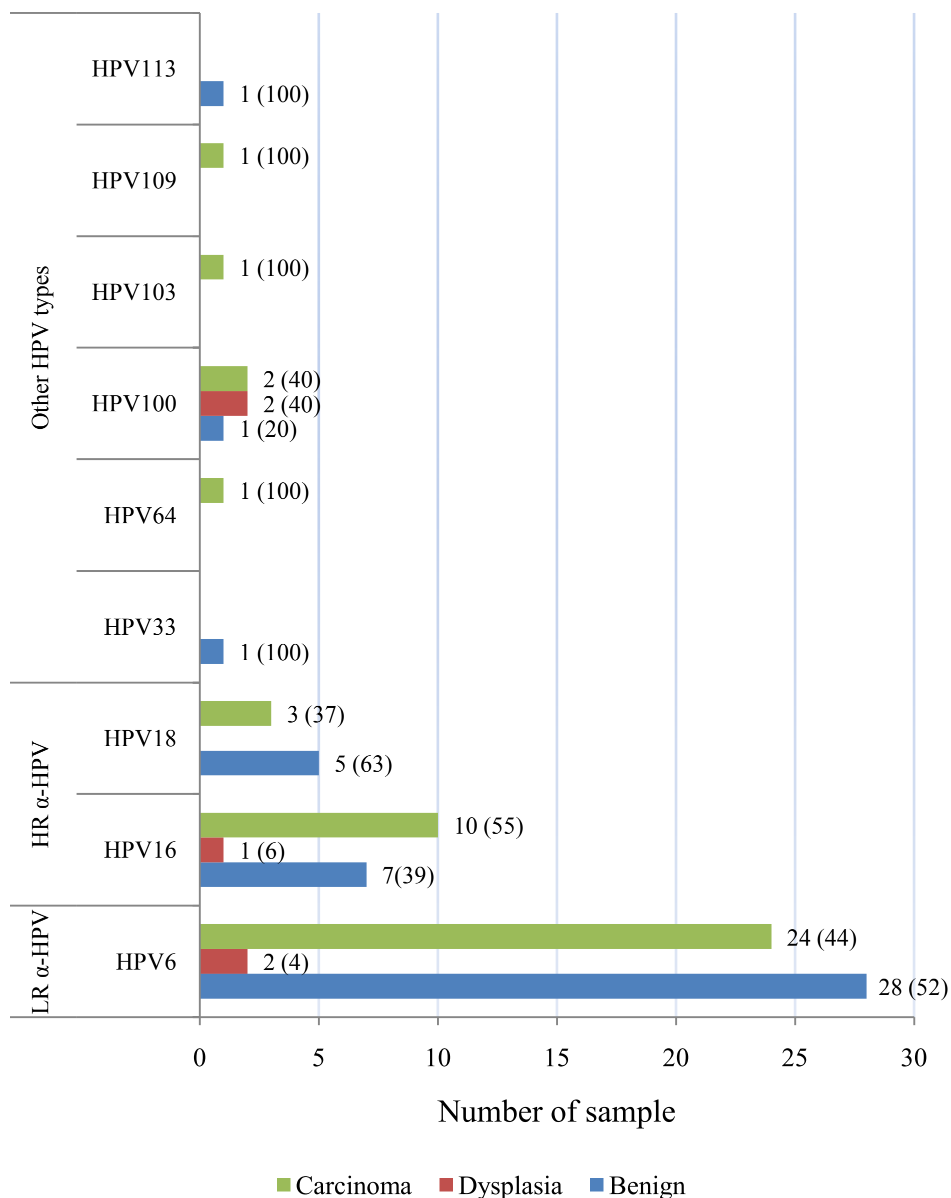



**Figure 4.3: The breakdown of specific HPV types (%) identified.** The percentage given is based on its total number from all the pathological groups

The distribution of HPV types within clinical groups for benign is shown in Table 4.6, dysplasia (Table 4.7), carcinoma (Table 4.8) and histopathological parameters for carcinoma in Table 4.9. It was observed that HPV16 and HPV6 were equally distributed between gender in benign and carcinoma. The latter type appeared in nearly similar proportions in carcinoma. The high risk HPV and other HPV types were predominantly

found in females compared to their counterpart in benign and carcinoma. Even though, HPV16 was only identified in male patients with dysplastic lesions but this difference relatively was not significant.

**Table 4.6: The distribution of HPV types within clinical groups in benign (*N* = 43)**

| Parameters | *N* | HR α-HPV (%) | | LR α-HPV (%) | Other HPV types (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HPV16 | HPV18 | HPV6 | HPV33 | HPV64 | HPV100 | HPV103 | HPV109 | HPV113 |
| **Gender:** | | | | | | | | | | |
| Female | 45 | 3 (6.7) | 5 (11.1) | 14 (31.1) | 1 (2.2) | - | - | - | - | 1 (2.2) |
| Male | 39 | 4 (10.3) | - | 14 (35.9) | - | - | 1 (2.6) | - | - | - |
| **Age group:** | | | | | | | | | | |
| 16 – 57y | 52 | 3 (5.8) | 2 (3.8) | 18 (34.6) | 1 (1.9) | - | 1 (1.9) | - | - | 1 (1.9) |
| 58-99y | 32 | 4 (12.5) | 3 (8.6) | 10 (31.3) | - | - | - | - | - | - |
| **Anatomic sites:** | | | | | | | | | | |
| Buccal mucosa | 41 | 3 (7.3) | 4 (9.8) | 15 (36.6) | - | - | 1 (2.4) | - | - | - |
| Floor of the mouth | 3 | - | - | 1 (33.3) | - | - | - | - | - | - |
| Gingivae | 6 | - | - | 2 (33.3) | - | - | - | - | - | - |
| Hard palate | 2 | - | - | - | - | - | - | - | - | - |
| Soft palate | 3 | - | - | 2 (66.7) | - | - | - | - | - | - |
| Upper lip | 2 | 1 (50.0) | - | - | - | - | - | - | - | - |
| Lower lip | 4 | 1 (25.0) | 1 (25.0) | - | - | - | - | - | - | - |
| Retromolar pad | 1 | - | - | 1 (100.0) | - | - | - | - | - | - |
| Skin | 1 | - | - | - | - | - | - | - | - | - |
| Tongue | 21 | 2 (9.5) | - | 7 (33.3) | 1 (4.8) | - | - | - | - | 1 (4.8) |
| **Disease status:** | | | | | | | | | | |
| No recurrence | 84 | 7 (8.3) | 5 (6.0) | 28 (33.3) | 1 (1.2) | - | 1 (1.2) | - | - | 1 (1.2) |
| Recurrence | - | - | - | - | - | - | - | - | - | - |
| **Disease outcome (*N*=0):** | | | | | | | | | | |
| Alive | - | - | - | - | - | - | - | - | - | - |
| Dead | - | - | - | - | - | - | - | - | - | - |
| **Subtotal** | | **7 (58.3)** | **5 (41.7)** | **28 (100.0)** | **1 (33.3)** | **-** | **1 (33.3)** | **-** | **-** | **1 (33.3)** |
| **TOTAL (*N* = 84)** | | **12 (14.3)** | | **28 (33.3)** | **3 (3.6)** | | | | | |

**Table 4.7: The distribution of HPV types within clinical groups in dysplasia (*N* = 5)**

| Parameters | *N* | HR α-HPV (%) | | LR α-HPV (%) | Other HPV types (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HPV16 | HPV18 | HPV6 | HPV33 | HPV64 | HPV100 | HPV103 | HPV109 | HPV113 |
| **Gender:** | | | | | | | | | | |
| Female | 5 | - | - | - | - | - | 1 (20.0) | - | - | - |
| Male | 7 | 1 (14.3) | - | 2 (28.6) | - | - | 1 (14.3) | - | - | - |
| **Age group:** | | | | | | | | | | |
| 16 – 57y | 2 | 1 (50.0) | - | - | - | - | 1 (50.0) | - | - | - |
| 58-99y | 10 | - | - | 2 (20.0) | - | - | 1 (10.0) | - | - | - |
| **Anatomic sites:** | | | | | | | | | | |
| Buccal mucosa | 1 | - | - | - | - | - | 1 (100.0) | - | - | - |
| Floor of the mouth | 8 | 1 (12.5) | - | 1 (12.5) | - | - | 1 (12.5) | - | - | - |
| Gingivae | - | - | - | - | - | - | - | - | - | - |
| Hard palate | 1 | - | - | - | - | - | - | - | - | - |
| Soft palate | 2 | - | - | 1 (50.0) | - | - | - | - | - | - |
| Upper lip | - | - | - | - | - | - | - | - | - | - |
| Lower lip | - | - | - | - | - | - | - | - | - | - |
| Retromolar pad | - | - | - | - | - | - | - | - | - | - |
| Skin | - | - | - | - | - | - | - | - | - | - |
| Tongue | - | - | - | - | - | - | - | - | - | - |
| **Disease status:** | | | | | | | | | | |
| No recurrence | 12 | 1 (8.3) | - | 2 (16.7) | - | - | 2 (16.7) | - | - | - |
| Recurrence | - | - | - | - | - | - | - | - | - | - |
| **Disease outcome (*N*=0):** | | | | | | | | | | |
| Alive | - | - | - | - | - | - | - | - | - | - |
| Dead | - | - | - | - | - | - | - | - | - | - |
| **Subtotal** | | **1 (100.0)** | **-** | **2 (100.0)** | **-** | **-** | **2 (100.0)** | **-** | **-** | **-** |
| **TOTAL (*N* = 12)** | | **1 (8.3)** | | **2 (16.7)** | **2 (16.7)** | | | | | |

**Table 4.8: The distribution of HPV types within clinical groups in carcinoma (*N* = 42)**

| Parameters | *N* | HR α-HPV (%) | | LR α-HPV (%) | Other HPV types (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HPV16 | HPV18 | HPV6 | HPV33 | HPV64 | HPV100 | HPV103 | HPV109 | HPV113 |
| **Gender:** | | | | | | | | | | |
| Female | 49 | 10 (20.4) | 1 (2.0) | 13 (26.5) | - | 1 (2.0) | - | 1 (2.0) | 1 (2.0) | - |
| Male | 31 | - | 2 (6.5) | 11 (35.5) | - | - | 2 (6.5) | - | - | - |
| **Age group:** | | | | | | | | | | |
| 16 – 57y | 38 | 9 (23.7) | 1 (2.6) | 8 (21.1) | - | - | 1 (2.6) | 1 (2.6) | 1 (2.6) | - |
| 58-99y | 42 | 1 (2.4) | 2 (4.8) | 16 (38.1) | - | 1 (2.4) | 1 (2.4) | - | - | - |
| **Anatomic sites:** | | | | | | | | | | |
| Buccal mucosa | 3 | - | - | 2 (66.7) | - | - | - | - | - | - |
| Floor of the mouth | 12 | 1 (8.3) | 1 (8.3) | 6 (50.0) | - | - | - | - | - | - |
| Gingivae | 4 | - | 1 (25.0) | 2 (50.0) | - | - | - | - | - | - |
| Hard palate | - | - | - | - | - | - | - | - | - | - |
| Soft palate | 34 | 9 (26.5) | - | 7 (20.6) | - | - | 1 (2.9) | 1 (2.9) | 1 (2.9) | - |
| Upper lip | - | - | - | - | - | - | - | - | - | - |
| Lower lip | 4 | - | - | 2 (50.0) | - | - | - | - | - | - |
| Retromolar pad | 3 | - | - | - | - | - | - | - | - | - |
| Skin | - | - | - | - | - | - | - | - | - | - |
| Tongue | 20 | - | 1 (5.0) | 5 (25.0) | - | 1 (5.0) | 1 (5.0) | - | - | - |
| **Disease status:** | | | | | | | | | | |
| No recurrence | 75 | 10 (13.3) | 3 (4.0) | 22 (29.3) | - | 1 (1.3) | 2 (2.7) | 1 (1.3) | 1 (1.3) | - |
| Recurrence | 5 | - | - | 2 (40.0) | - | - | - | - | - | - |
| **Disease outcome (*N*=80):** | | | | | | | | | | |
| Alive | 45 | 10 (22.2) | 1 (2.2) | 10 (22.2) | - | - | 1 (2.2) | 1 (2.2) | 1 (2.2) | - |
| Dead | 35 | - | 2 (5.7) | 14 (40.0) | - | 1 (2.9) | 1 (2.9) | - | - | - |
| **Subtotal** | | **10 (76.9)** | **3 (23.1)** | **24 (100.0)** | **-** | **1 (20.0)** | **2 (40.0)** | **1 (20.0)** | **1 (20.0)** | **-** |
| **TOTAL (*N* = 80)** | | **13 (16.3)** | | **24 (30.0)** | | | | **5 (6.3)** | | |

**Table 4.9: The distribution of HPV types within histopathological parameters in carcinoma group (*N* = 42)**

| Parameters | *N* | HR α-HPV (%) | | LR α-HPV (%) | Other HPV types (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HPV16 | HPV18 | HPV6 | HPV33 | HPV64 | HPV100 | HPV103 | HPV109 | HPV113 |
| **Tumour size:** | | | | | | | | | | |
| $T_1$ | 41 | 10 (24.4) | 1 (2.4) | 9 (22.0) | - | - | - | 1 (2.4) | 1 (2.4) | - |
| $T_2$ | 22 | - | 1 (4.5) | 6 (27.3) | - | 1 (4.5) | - | - | - | - |
| $T_3$ | 13 | - | - | 7 (53.8) | - | - | 2 (15.4) | - | - | - |
| $T_4$ | 4 | - | 1 (25.0) | 2 (50.0) | - | - | - | - | - | - |
| **Regional Lymph Nodes:** | | | | | | | | | | |
| $N_0$ | 67 | 10 (14.9) | 1 (1.5) | 17 (25.4) | - | 1 (1.5) | 2 (3.0) | 1 (1.5) | 1 (1.5) | - |
| $N_1$ | 7 | - | 1 (14.3) | 3 (42.9) | - | - | - | - | - | - |
| $N_2$ | 6 | - | 1 (16.7) | 4 (66.7) | - | - | - | - | - | - |
| **Histological grade:** | | | | | | | | | | |
| Well differentiated | 9 | - | - | 2 (22.2) | - | - | - | - | - | - |
| Moderately differentiated | 58 | 10 (17.2) | 2 (3.4) | 15 (25.9) | - | 1 (1.7) | - | 1 (1.7) | 1 (1.7) | - |
| Poorly differentiated | 11 | - | 1 (9.1) | 6 (54.5) | - | - | 2 (18.2) | - | - | - |
| Basaloid | 2 | - | - | 1 (50.0) | - | - | - | - | - | - |
| **Subtotal** | | **10 (76.9)** | **3 (23.1)** | **24 (100.0)** | **-** | **1 (20.0)** | **2 (40.0)** | **1 (20.0)** | **1 (20.0)** | **-** |
| **TOTAL (*N* = 80)** | | **13 (16.3)** | | **24 (30.0)** | **5 (6.3)** | | | | | |

The distribution of high risk HPV types was comparable across age groups in benign tumours. This was not the case for carcinoma where it was more abundant in the younger groups although it has been previously demonstrated that the total frequency of high risk HPV types was similar in both pathological groups. HPV6, on the other hand, did not follow a similar distribution where in younger patients it was higher in benign tumours but lower in carcinoma compared to their counterparts.

We have to admit at this point the difficulties in drawing very conclusive evidence on the prevalence of HPV based on subsite analysis in each pathological group. Since there was a very uneven distribution of the available samples observed in each anatomic site, we focused on the sites which were significantly higher in representative samples for meaningful comparison. Therefore, within and between pathological groups, comparison could be made possible using the above criteria. In benign tumours for instance, buccal mucosa ($n$=41) and tongue ($n$=21) were the two more prominent subsites. Floor of the mouth ($n$=12), soft palate ($n$=34) and tongue ($n$=20) were significantly higher in carcinoma. Since we identified only a single candidate available for dysplasia which was floor of the mouth ($n$=8), comparing with its counterpart in carcinoma could be useful. Tongue was the suitable anatomic site to compare between benign and carcinoma.

In benign tissue, HPV6 was commonly present in most of the sites but was highest in buccal mucosa ($n$=15). The high risk HPV types were detected in four subsites, upper lip, lower lip, tongue and similarly buccal mucosa was the highest ($n$=7). For other HPV types, two were identified in the tongue, HPV33 and HPV113 and one in

buccal mucosa, HPV100. Only one candidate each, HPV16, HPV6 and HPV100 was observed in the floor of the mouth of dysplasia patients.

The last three clinicopathological parameters, tumour size, regional lymph nodes and histological grade are only applicable for carcinoma group (Table 4.9). All HPV16 was only detected in patient's tissue with 2 cm or less tumour size ($T_1$) and no regional lymph node metastasis ($N_0$). In addition, the majority of HPVs were identified in patient's tissue with moderately differentiated. Although HPV6 DNA was amplified in all subcategories of clinicopathological parameters concerned, it uniquely demonstrated the highest frequency in which HPV16 was present. HPV18, on the other hand, did not follow the pattern as such. Thus far, no association was found between typical pattern of HPV distribution and specific HPV type.

Pearson Chi-squared tests were carried out in each clinicopathological groups (benign, dysplasia and carcinoma) using the frequency of HPV positivity to determine if there is a statistically significant relationship. Phi value (Φ) measures the size effect and for 2x2 cross-tabulation with nominal data ($df$=1) and Cramer's V for larger crosstabs ($df$=$n$-1), $n$ is the number of variable. Fisher's Exact Test was utilised to interpret the results for small samples in which chi-squared would be violated. This is the case where more than 20% of the cells have expected frequencies less than 5 (Coakes and Steed, 2000).

There were no significant differences observed in overall HPV status (Appendix 44), α-HPV status (Appendix 45) and LR α-HPV status (Appendix 46) in all clinical parameters tested. Unlike previous observations, there were significant differences in

the frequency of HR α-HPV status (Appendix 47) and mostly in carcinoma samples for age group ($p<0.05$), disease status ($p<0.05$), DNA purity ($p<0.01$) and tumour size ($p<0.05$). Similarly, in benign group this difference was observed only in DNA purity ($p<0.05$). In addition, this frequency appeared comparable in dysplasia group. The phi value observed ranging from 0.224 to 0.332 and thus the effect of size is considered to be small to medium according to Cohen (1988).

## 4.8 ODDS RATIO ANALYSIS AMONG DEMOGRAPHIC AND CLINICAL PARAMETERS BY HPV STATUS

The analysis of demographic, and clinical (Table 4.10 and Table 4.11) and histopathological risk factor characteristics (Table 4.12 and Table 4.13) of oral cancer in each pathological group by overall HPV types and high risk HPV types status were carried out. Histopathological risk factor characteristics were applicable only for the carcinoma group. It was observed that a statistically significant estimate of odds ratio was observed in relation to high risk HPV status for certain parameters but this was not the case for overall HPV types for all parameters. In the high risk HPV group, benign and carcinoma patients had significant less relative risk in males compared to their counterpart [0.2 (0.1-0.5), $p<0.001$ for benign and 0.3 (0.2-0.6), $p<0.001$ for carcinoma group]. Similarly for age group, older patients had significant less relative risk compared to reference group [0.1 (0.1-0.3), $p<0.001$ for benign and 0.4 (0.2-0.7), $p<0.001$for carcinoma]. For disease status and disease outcome, only carcinoma group had significant less relative risk compared to their counterpart [for disease recurrence and for living patients]

**Table 4.10: Demographic and risk factor characteristics of oral cancer by HPV status in each pathological group**

| Parameter | *N* | Benign (*N*=84) | | | | Dysplasia (*N*=12) | | | | Carcinoma (*N*=80) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HPV- (%) | HPV+ (%) | OR (95%CI) | *p* value | HPV- (%) | HPV+ (%) | OR (95%CI) | *p* value | HPV- (%) | HPV+ (%) | OR (95%CI) | *p* value |
| Gender | | | | | | | | | | | | | |
| Female$^{\alpha}$ | 99 | 21 (51.2) | 24 (55.8) | 1.0 | 0.673 | 4 (57.1) | 1 (20.0) | 1.0 | 0.215 | 22 (57.9) | 27 (64.3) | 1.0 | 0.476 |
| Male | 77 | 20 (48.8) | 19 (44.2) | 0.8 (0.4-2.0) | | 3 (42.9) | 4 (80.0) | 0.3 (0.0-2.2) | | 16 (42.1) | 15 (35.7) | 1.2 (0.7-2.2) | |
| Age group | | | | | | | | | | | | | |
| 16-57y$^{\alpha}$ | 92 | 26 (63.4) | 26 (60.5) | 1.0 | 0.781 | 0(0) | 2 (40.0) | N/E | 0.999 | 17 (44.7) | 21 (50.0) | 1.0 | 0.517 |
| 58-99y | 84 | 15 (36.6) | 17 (39.5) | 1.1 (0.5-2.7) | | 7 (100.0) | 3 (60.0) | | | 21 (55.3) | 21 (50.0) | 1.2 (0.7-2.3) | |
| Anatomic site | | | | | | | | | | | | | |
| Buccal mucosa$^{\alpha}$ | 45 | 18 (43.9) | 23 (53.5) | 1.0 | 0.995 | 0 (0) | 1 (20.0) | 1.0 | 0.921 | 1 (2.6) | 2 (4.8) | 1.0 | 0.812 |
| Floor of the mouth | 23 | 2 (4.9) | 1 (2.3) | 0.4 (0.0-4.7) | 0.458 | 5 (71.4) | 3 (60.0) | N/E | | 4 (10.5) | 8 (19.0) | 2.0 (0.2-22.1) | 0.571 |
| Gingivae | 10 | 4 (9.8) | 2 (4.7) | 0.4 (0.1-2.4) | 0.309 | 0 (0) | 0 (0) | | | 1 (2.6) | 3 (7.1) | 2.0 (0.6-6.6) | 0.258 |
| Hard palate | 3 | 2 (4.9) | 0 (0) | N/E | 0.999 | 1 (14.3) | 0 (0) | 0.6 (0.1-2.5) | 0.484 | 0 (0) | 0 (0) | | |
| Soft palate | 39 | 1 (2.4) | 2 (4.7) | 1.6 (0.1-18.7) | 0.723 | 1 (14.3) | 1 (20.0) | N/E | | 15 (39.5) | 19 (45.2) | 3.0 (0.3-28.8) | 0.341 |
| Upper lip | 2 | 1 (2.4) | 1 (2.3) | 0.8 (0.0-13.4) | 0.866 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | |
| Lower lip | 8 | 2 (4.9) | 2 (4.7) | 0.8 (0.1-6.1) | 0.815 | 0 (0) | 0 (0) | | | 2 (5.3) | 2 (4.8) | 1.3 (0.6-2.5) | 0.494 |
| Retromolar pad | 4 | 0 (0) | 1 (2.3) | N/E | 1.000 | 0 (0) | 0 (0) | | | 3 (7.9) | 0 (0) | 1.0 (0.1-7.1) | 1.000 |
| Skin | 1 | 1 (2.4) | 0 (0) | N/E | 1.000 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | |
| Tongue | 41 | 10 (24.4) | 11 (25.6) | 0.9 (0.3-2.5) | 0.781 | 0 (0) | 0 (0) | | | 12 (31.6) | 8 (19.0) | N/E | 0.999 |
| Disease status | | | | | | | | | | | | | |
| No recurrence$^{\alpha}$ | 171 | 41 (100.0) | 43 (100.0) | N/A | N/A | 7 (100.0) | 5 (100.0) | N/A | N/A | 35 (92.1) | 40 (95.2) | 1.0 | 0.564 |
| Recurrence | 5 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | | 3 (7.9) | 2 (4.8) | 1.1 (0.7-1.8) | |
| Disease outcome | | | | | | | | | | | | | |
| Alive$^{\alpha}$ | 45 | 0 (0) | 0 (0) | N/A | N/A | 0 (0) | 0 (0) | N/A | N/A | 21 (55.3) | 24 (57.1) | 1.1 (0.5-2.1) | 0.866 |
| Dead | 35 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | | 17 (44.7) | 18 (42.9) | 1.0 | |

$^{\alpha}$ – reference group; N/A – not available; N/E not estimable

**Table 4.11: Demographic and risk factor characteristics of oral cancer by high risk-HPV status in each pathological group**

| Parameter | *N* | Benign (*N* = 84) | | | | Dysplasia (*N* = 12) | | | | Carcinoma (*N* = 80) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | hrHPV- (%) | hrHPV+ (%) | OR (95%CI) | *p* value | hrHPV- (%) | hrHPV+ (%) | OR (95%CI) | *p* value | hrHPV- (%) | hrHPV+ (%) | OR (95%CI) | *p* value |
| Gender | | | | | | | | | | | | | |
| Female[α] | 99 | 37 (52.9) | 8 (57.1) | 1.0 | 0.769 | 5 (45.5) | 0 (0) | N/E | 0.999 | 38 (56.7) | 11 (84.6) | 1.0 | 0.000 |
| Male | 77 | 33 (47.1) | 6 (42.9) | 0.8 (0.3-2.7) | | 6 (54.5) | 1 (100.0) | | | 29 (43.3) | 2 (15.4) | 0.3 (0.2-0.6) | |
| Age group | | | | | | | | | | | | | |
| 16-57y[α] | 92 | 47 (67.1) | 5 (35.7) | 1.0 | 0.034 | 1(9.1) | 1 (100.0) | 1.0 (0.1-16.0) | 1.000 | 28 (41.8) | 10 (76.9) | 1.0 | 0.005 |
| 58-99y | 84 | 23 (32.9) | 9 (64.3) | 3.7 (1.1-12.2) | | 10 (90.9) | 0 (0) | | | 39 (58.2) | 3 (23.1) | 0.4 (0.2-0.7) | |
| Anatomic site | | | | | | | | | | | | | |
| Buccal mucosa[α] | 45 | 33 (47.1) | 8 (57.1) | 1.0 | 0.892 | 1 (9.1) | 0 (0) | 1.0 | 0.346 | 3 (4.5) | 0 (0) | 1.0 | 0.059 |
| Floor of the mouth | 23 | 3 (4.3) | 0 (0) | | 0.999 | 7 (63.6) | 1 (100.0) | N/E | | 10 (14.9) | 2 (15.4) | N/E | 0.999 |
| Gingivae | 10 | 5 (7.1) | 1 (7.1) | 0.8 (0.1-8.1) | 0.869 | 0 (0) | 0 (0) | | | 3 (4.5) | 1 (7.7) | 0.2 (0-0.9) | 0.038 |
| Hard palate | 3 | 2 (2.9) | 0 (0) | | 0.999 | 1 (9.1) | 0 (0) | 0.1 (0-1.2) | 0.069 | 0 (0) | 0 (0) | | |
| Soft palate | 39 | 3 (4.3) | 0 (0) | | 0.999 | 2 (18.2) | 0 (0) | N/E | | 25 (37.3) | 9 (69.2) | 0.3 (0-3.2) | 0.341 |
| Upper lip | 2 | 1 (1.4) | 1 (7.1) | 4.1 (0.2-73.3) | 0.334 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | |
| Lower lip | 8 | 2 (2.9) | 2 (14.3) | 4.1 (0.5-33.9) | 0.187 | 0 (0) | 0 (0) | | | 4 (6.0) | 0 (0) | 0.4 (0.2-0.8) | 0.009 |
| Retromolar pad | 4 | 1 (1.4) | 0 (0) | | 1.000 | 0 (0) | 0 (0) | | | 3 (4.5) | 0 (0) | N/E | 0.999 |
| Skin | 1 | 1 (1.4) | 0 (0) | | 1.000 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | |
| Tongue | 41 | 19 (27.1) | 2 (14.3) | 0.4 (0.1-2.3) | 0.321 | 0 (0) | 0 (0) | | | 19 (28.4) | 1 (7.7) | N/E | 0.999 |
| Disease status | | | | | | | | | | | | | |
| No recurrence[α] | 171 | 70 (100.0) | 14 (100.0) | N/A | N/A | 11 (100) | 1 (100) | N/A | N/A | 62 (95.5) | 13 (100.0) | 1.0 | 0.000 |
| Recurrence | 5 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | | 5 (7.5) | 0 (0) | 0.2 (0.1-0.4) | |
| Disease outcome | | | | | | | | | | | | | |
| Alive | 45 | 0 (0) | 0 (0) | N/A | N/A | 0 (0) | 0 (0) | N/A | N/A | 34 (50.7) | 11(84.6) | 0.1 (0-0.3) | 0.000 |
| Dead[α] | 35 | 0 (0) | 0 (0) | | | 0 (0) | 0 (0) | | | 33 (49.3) | 2 (15.4) | 1.0 | |

[α] – reference group; N/A – not available; N/E not estimable

## Table 4.12: Histopathological risk factor properties by HPV status in carcinoma group

| Parameter | *N* | Carcinoma (*N* = 80) | | | |
|---|---|---|---|---|---|
| | | HPV- (%) | HPV+ (%) | OR (95%CI) | *p* value |
| Tumour size | | | | | |
| $T_1$ (<2 cm)$^{\alpha}$ | 41 | 19 (50.0) | 22 (52.4) | 1.0 | 0.304 |
| $T_2$ (2-4 cm) | 22 | 14 (36.8) | 8 (19.0) | 1.2 (0.6-2.1) | 0.640 |
| $T_3$ (>4 cm) | 13 | 4 (10.5) | 9 (21.4) | 0.6 (0.2-1.4) | 0.207 |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | 1 (2.6) | 3 (7.1) | 2.3 (0.7-7.3) | 0.177 |
| Regional Lymph Nodes | | | | | |
| $N_0$$^{\alpha}$ | 67 | 34 (89.5) | 33 (78.6) | 1.0 | 0.925 |
| $N_1$ | 7 | 3 (7.9) | 4 (9.5) | 1.0 (0.6-1.6) | 0.903 |
| $N_2$ | 6 | 1 (2.6) | 5 (11.9) | 1.3 (0.3-6.0) | 0.706 |
| Histological grade | | | | | |
| Well differentiated$^{\alpha}$ | 9 | 7 (18.4) | 2 (4.8) | 1.0 | 0.287 |
| Moderately differentiated | 58 | 28 (73.7) | 30 (71.4) | 1.0 (0.1-16.0) | 1.000 |
| Poorly differentiated | 11 | 2 (5.3) | 9 (21.4) | 4.5 (1.0-21.0) | 0.054 |
| Basaloid | 2 | 1 (2.6) | 1 (2.4) | 1.1 (0.6-0.18) | 0.793 |

$^{\alpha}$ – reference group for odds ratio determination; N/A – not available; N/E not estimable

## Table 4.13: Histopathological risk factor properties by high risk HPV status in carcinoma group

| Parameter | *N* | Carcinoma (*N* = 80) | | | |
|---|---|---|---|---|---|
| | | hrHPV- (%) | hrHPV+ (%) | OR (95%CI) | *p* value |
| Tumour size | | | | | |
| $T_1$ (<2 cm)$^{\alpha}$ | 41 | 30 (44.8) | 11 (84.6) | 1.0 | 0.001 |
| $T_2$ (2-4 cm) | 22 | 21 (31.3) | 1 (7.7) | 0.4 (0.2-0.7) | 0.004 |
| $T_3$ (>4 cm) | 13 | 13 (19.4) | 0 (0) | 0.6 (0-0.4) | 0.003 |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | 3 (4.5) | 1 (7.7) | N/E | 0.998 |
| Regional Lymph Nodes | | | | | |
| $N_0$$^{\alpha}$ | 67 | 56 (83.6) | 11 (84.6) | 1.0 | 0.000 |
| $N_1$ | 7 | 6 (9.0) | 1 (7.7) | 1.0 (0.2-0.4) | 0.000 |
| $N_2$ | 6 | 5 (7.5) | 1 (7.7) | 1.3 (0-1.4) | 0.097 |
| Histological grade | | | | | |
| Well differentiated$^{\alpha}$ | 9 | 9 (13.4) | 0 (0) | 1.0 | 0.000 |
| Moderately differentiated | 58 | 46 (68.7) | 1 (7.7) | N/E | 0.999 |
| Poorly differentiated | 11 | 10 (14.9) | 12 (92.3) | 0.1 (0-0.8) | 0.028 |
| Basaloid | 2 | 2 (3.0) | 0 (0) | 0.3 (0.1-0.5) | 0.000 |

$^{\alpha}$ – reference group for odds ratio determination; N/A – not available; N/E not estimable

## 4.9 CORRELATION BETWEEN DEMOGRAPHIC AND CLINICAL PARAMETERS WITH HPV STATUS

The correlation analysis was undertaken to look for any association between clinical parameters with HPV status in benign (Appendix 48) dysplasia (Appendix 49) and carcinoma (Appendix 50). In each pathological group, comparison were individually made based on sub-categories of HPV group i.e. overall HPV status, α-HPV status, LR-HPV and HR-HPV.

No significant association was observed in benign and dysplasia for overall HPV, α-HPV, LR-HPV status. HR-HPV on the other hand, demonstrated significant negatively association for DNA purity in benign ($r$=-0.224, $p$<0.05) and age group in dysplasia ($r$=-0.674, $p$<0.05).

Most of the associations between HPV status and the parameters concerned were found in carcinoma patients. HR-HPV status for instance, was positively associated with disease status ($r$=0.252, $p$<0.05) and DNA purity ($r$=-0.332, $p$<0.01) and yet, negatively correlated with age group ($r$=-0.260, $p$<0.05). Moreover, significant association observed between α-HPV/LR-HPV status and regional lymph nodes, ($r$=0.227, $p$<0.05) and ($r$=-0.251, $p$<0.05), respectively. Only single correlation observed in this group to positively linked between LR-HPV with tumour size ($r$=0.242, $p$<0.05) but exhibited negative association between overall HPV and histological grade ($r$=-0.250, $p$<0.05).

## 4.10 SURVIVAL ANALYSIS (*N*=80)

Survival was measured in months from the date of diagnosis until death or until the patient was last known to be alive. The Kaplan-Meier curve of survival followed by Log Rank (Mantel-Cox) test was used to investigate whether there was an association between the clinical parameters and patients survival. All survival curves were generated in SPSS ver.16. Continuous data values were divided by the median and comparisons were made using the log-rank test. Since the above technique is the univariate version of survival analysis, later by performing Cox regression analysis it will further assisting the actual effect of confounders on the survival of the patient.

Cox proportional hazard models were used to assess the relationships between HPV and clinical/histopathological characteristics and to adjust for factors previously found to be prognostically significant in oral cancer. These analyses included gender, age group, anatomic site, tumour site, regional lymph nodes, histological grade, disease recurrence and HPV status also were examined in separate models. All variables were assessed for the proportional hazard assumption before inclusion in the models. The magnitude of the associations was assessed with hazard ratios (HRs) calculated from the Cox regression models. Statistically significance was based on two-tailed tests and p-values $\leq 0.05$. Statistical analyses were performed using SPSS 16.0.

### 4.10.1 Kaplan-Meier Survival Analysis

The results suggested that the overall patient survival was worst in six groups (Table 4.14):

1. Male compared with female ($p$<0.01) (Figure 4.4).
2. Older patients age 58 to 99 ($p$<0.01) (Figure 4.5).
3. Anatomic site ($p$<0.01) (Figure 4.6).
4. Tumour size ($p$<0.01) (Figure 4.7).
5. Regional lymph nodes ($p$<0.01) (Figure 4.8).
6. Histological grade (p<0.01) (Figure 4.9).

**Table 4.14: The prognostic significance from overall survival analysis in carcinoma patients**

| Parameter (*N*=80) | Log Rank (Mantel-Cox) | | |
|---|---|---|---|
| | *Chi-Square* | *df* | *Sig.* |
| Gender | 11.869 | 1 | 0.001** |
| Age group | 14.055 | 1 | 0.000** |
| Anatomic site | 29.181 | 6 | 0.000** |
| Tumour size | 38.353 | 3 | 0.000** |
| Regional Lymph Nodes | 18.751 | 2 | 0.000** |
| Histological grade | 16.088 | 3 | 0.001** |
| Recurrence | 0.395 | 1 | 0.530 |
| HPV status | 0.002 | 1 | 0.963 |
| α-HPV status | 0.014 | 1 | 0.906 |
| High risk HPV | 3.915 | 1 | 0.048* |
| Low risk HPV | 3.561 | 1 | 0.059 |
| HPV16 status | 6.918 | 1 | 0.009** |
| Other HPV types | 0.021 | 1 | 0.885 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

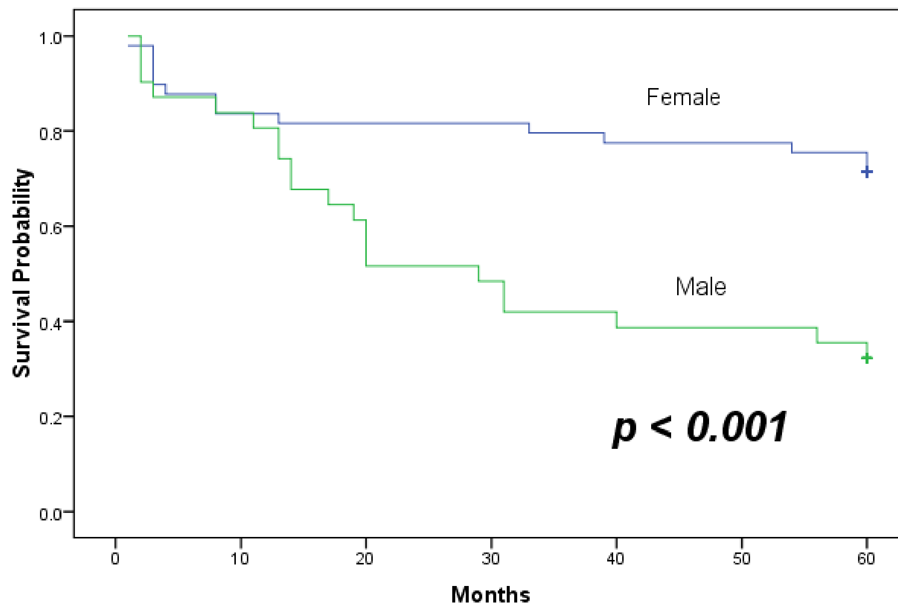



**Figure 4.4: An association between gender and overall survival as generated by Kaplan-Meier test.** Vertical tick marks on curves indicate censored observations.

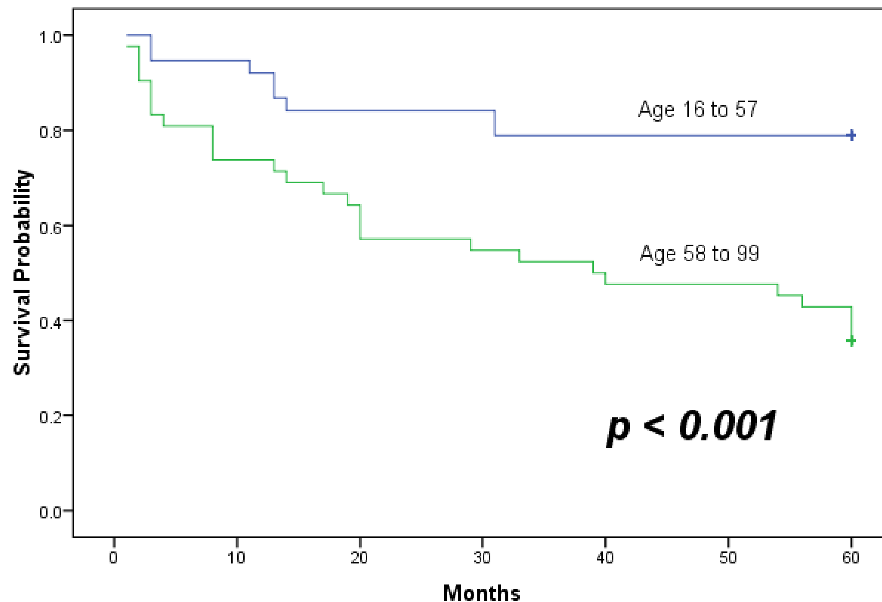



**Figure 4.5: An association between age group and overall survival as generated by Kaplan-Meier test.**

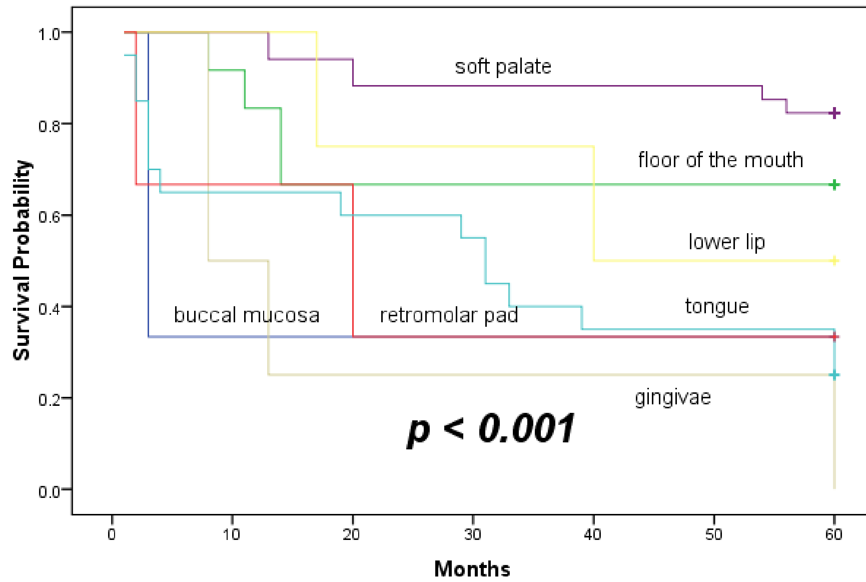



**Figure 4.6: An association among anatomic sites and overall survival as generated by Kaplan-Meier test.**

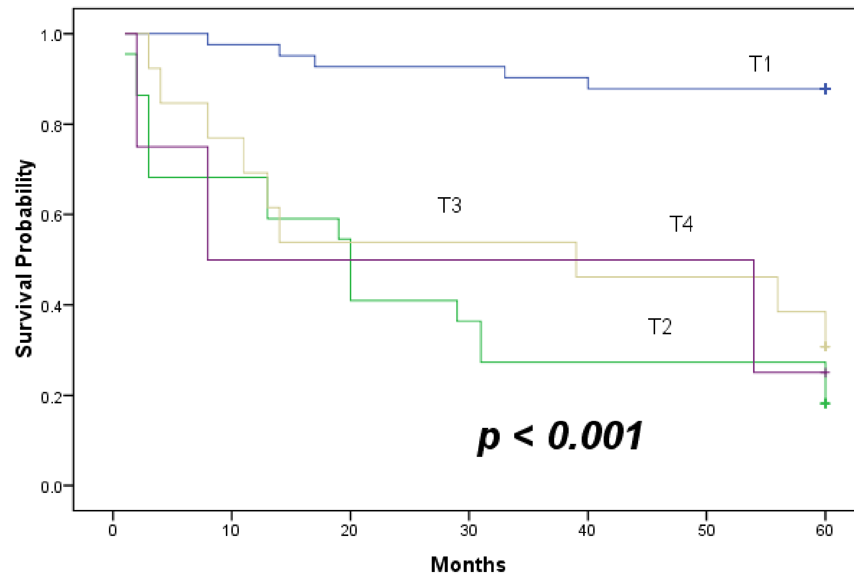



**Figure 4.7: An association among tumour size and overall survival as generated by Kaplan-Meier test.**

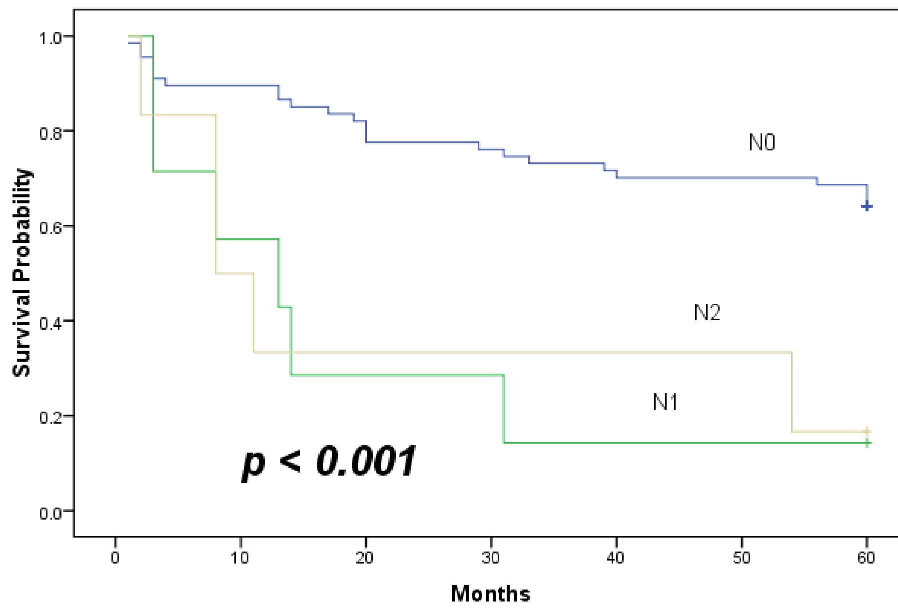



**Figure 4.8: An association among regional lymph nodes and overall survival as generated by Kaplan-Meier test.**

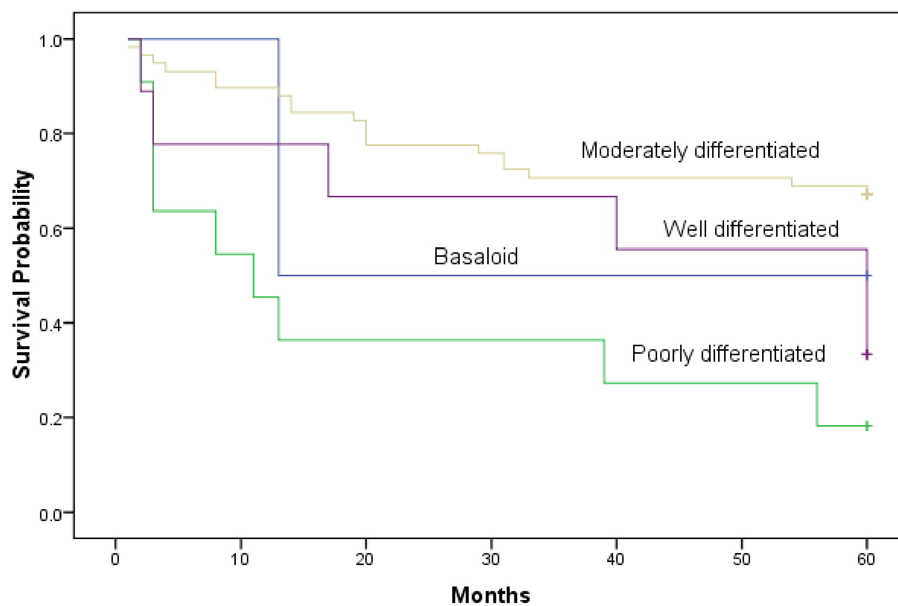



**Figure 4.9: An association among histological grade and overall survival as generated by Kaplan-Meier test.**

### 4.10.2 Cox Regression Model

**Table 4.15: The summary of the survival analysis done by Cox regression**

| Parameter ($N$=80) | Cox Regression Model | | |
|---|---|---|---|
| | *Wald* | *df* | *Sig.* |
| Gender | 10.4888 | 1 | 0.001** |
| Age group | 11.763 | 1 | 0.001** |
| Anatomic site | 21.830 | 6 | 0.001** |
| Tumour size | 24.560 | 3 | 0.000** |
| Regional Lymph Nodes | 15.469 | 2 | 0.000** |
| Histological grade | 13.598 | 3 | 0.004** |
| Recurrence | 0.381 | 1 | 0.537 |
| HPV status | 0.002 | 1 | 0.963 |
| α-HPV status | 0.014 | 1 | 0.907 |
| High risk HPV | 3.312 | 1 | 0.069 |
| Low risk HPV | 3.363 | 1 | 0.067 |
| HPV16 status | 2.699 | 1 | 0.100 |
| Other HPV types | 0.021 | 1 | 0.886 |

$N$ – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

## 4.11 CONCLUSIONS

Our results thus far from this cohort could conclude:

**1. DNA extraction from 183 FFPETs**

- The DNA extraction method that had been established in the pilot study was employed in this bigger cohort and successfully isolated DNA from 183 FFPETs.
- No significant difference was observed in relation to DNA yield and DNA purity amongst oral benign lesions, dysplasia, carcinoma and normal samples.

2. **PCR amplification for L1 HPV detection**

- The first round of an initial DNA quality screening of FFPETs (as assessed by the amplification of the β-globin fragment by PCR) was 168/183 (91.8%), by repeating the process with the initial failures (15 samples) it finally increased the success rate to 100%.
- Regardless of the pathological group, we successfully amplified 137/176 (77.8%) of presumptive positive L1 HPV fragment by PCR.

3. **Purification and DNA sequencing of the PCR products**

- All 137 fragments were successfully purified and most of them by using a Gel Extraction Kit.
- DNA sequencing of the shortest fragment, 140bp and multiple DNA sequence alignment confirmed the true L1 HPV amplicons were 90/137 (65.7%).
- The justification not to omit 47 amplicons (which were finally identified as false positive PCR products) was due to the fact they resembled the expected amplicon size but had lower PCR band intensity, compared to the positive control.

4. **HPV prevalence in benign, dysplasia, carcinoma and normal samples**

- The distribution of HPV detected in each pathological group was 43/84 (51.2%) in benign, 5/12 (41.7%) in dysplasia, 42/80 (52.5%) in carcinoma and 1/7 (14.3%) in normal samples tested.
- Overall HPV positivity, their prevalence was not statistically significant different amongst pathological groups.
- A comparison based on HPV type specific demonstrated very significant differences especially for high risk HPV and HPV6.

**5. Determination of an association between HPV status and disease progression**

- Across the pathological groups from benign to carcinoma, generally we found an increase in significant association between HPV status and demographic/clinical parameters.
- At this point, we could deduce a generalisation by stating the relatedness of HPVs status on oral disease progression.
- Despite dominating all the associations regardless of the pathological groups, HR-HPV appeared not to have any connection with clinical parameters.
- If there was HPV-related case to clinical parameters, the candidate was LR-HPV.

**6. Potential association with survival by HPV status**

- Kaplan-Meier test revealed that patient survival was worst in male, younger individuals, carcinoma located at the gingivae, $T_2$ tumour size (2-4 cm), $N_1$ nodes and poorly differentiated tissues and these were confirmed by Cox Regression analysis.
- Unfortunately, none of the factors could be related to HPV status.

# CHAPTER 5

# p16 AND ORAL DISEASE PROGRESSION

## 5.1 OVERVIEW

An overview of the aims of this chapter is given as follows:

- To investigate the expression of p16 of the benign oral lesions, dysplasias and carcinomas.
- To investigate the p16 status, p16 staining intensity, demographic and clinicopathological parameters by comparison of the benign, dysplasia and carcinoma samples.
- To investigate the correlation among demographic and clinical parameters associated with p16.
- To investigate the potential association with survival for p16 (for carcinoma group since survival data was available for this group only).

## 5.2 CLINICAL DATA

A similar cohort of 183 cases with histologically confirmed benign, dysplasia and squamous cell carcinoma of various anatomic sites (which were previously utilised for HPV genotyping) was initially used in this study. The clinical details of 183 samples from those patients were previously described in appropriate section of Chapter 4. Unfortunately, two samples (NS02007899 1 and NS02008655 1A) of the benign group were excluded from this study due to insufficient tissue to be analysed. As a result, 82 samples were available for p16 expression study in this group. Therefore, the total number in this cohort was 181 (Table 5.1).

**Table 5.1: Clinical details of oral cancer patients**

| Characteristic | *N* | Benign *N* (%) | Dysplasia *N* (%) | Carcinoma *N* (%) |
|---|---|---|---|---|
| Total | 174 | 82 (47.1) | 12 (6.9) | 80 (46.0) |
| Gender | | | | |
| Female | 97 | 43 (44.3) | 5 (5.2) | 49 (50.5) |
| Male | 77 | 39 (50.6) | 7 (9.1) | 31 (40.3) |
| Age group[1] (range, 16-99y) | | | | |
| 16-57y | 90 | 50 (55.6) | 2 (2.2) | 38 (42.2) |
| 58-99y | 84 | 32 (38.1) | 10 (11.9) | 42 (50.0) |
| Anatomic site | | | | |
| Buccal mucosa | 45 | 41 (91.1) | 1 (2.2) | 3 (6.7) |
| Floor of the mouth | 23 | 3 (13.0) | 8 (34.8) | 12 (52.2) |
| Gingivae | 9 | 5 (55.6) | 0 (0.0) | 4 (44.4) |
| Hard palate | 3 | 2 (66.7) | 1 (33.3) | 0 (0.0) |
| Soft palate | 39 | 3 (7.7) | 2 (5.1) | 34 (87.2) |
| Upper lip | 2 | 2 (100.0) | 0 (0.0) | 0 (0.0) |
| Lower lip | 8 | 4 (50.0) | 0 (0.0) | 4 (50.0) |
| Retromolar pad | 4 | 1 (25.0) | 0 (0.0) | 3 (75.0) |
| Skin | 1 | 1 (100.0) | 0 (0.0) | 0 (0.0) |
| Tongue | 40 | 20 (50.0) | 0 (0.0) | 20 (50.0) |
| Disease status | | | | |
| No recurrence | 169 | 82 (48.5) | 12 (7.1) | 75 (44.4) |
| Recurrence | 5 | 0 (0.0) | 0 (0.0) | 5 (100.0) |
| Disease outcome (*N*=80) | | | | |
| Alive | 45 | 0 (0.0) | 0 (0.0) | 45 (100.0) |
| Dead | 35 | 0 (0.0) | 0 (0.0) | 35 (97.2) |

*Note: N, number of patients [1] Age group was based on median age*

## 5.3 RESULTS

A representative of tissue specimens positively stained with the p16 antibody in the benign, dysplastic and carcinoma as compared with normal tissue are given in Figure 5.1. The result of staining of all the normal tissues with anti-p16 antibody was negative. All p16 IHC slides were semi-quantitatively assessed as was described in Chapter 2 in which p16 positivity and p16 staining intensity were taken into account. As expected, variation exists within and between pathological groups in relation to p16 status, nuclear and/or cytoplasmic staining was considered for the simplicity of the p16 positivity and intensity determination.

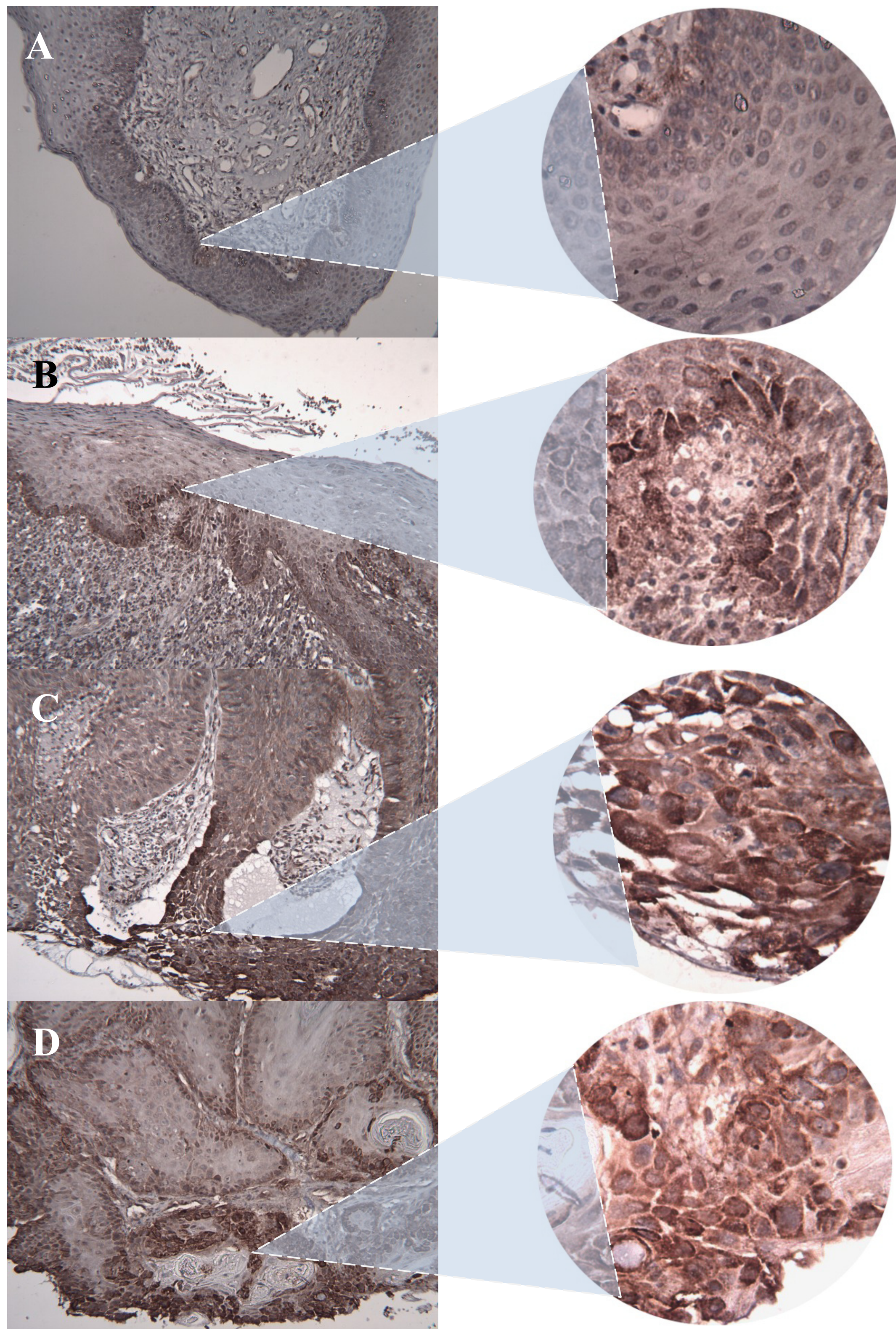



**Figure 5.1: Photomicrograph of p16 staining across pathological groups.** (A) Normal, (B) Benign, (C) Dysplasia and (D) Carcinoma at x100 magnification (rectangular) and x400 magnification (circle)

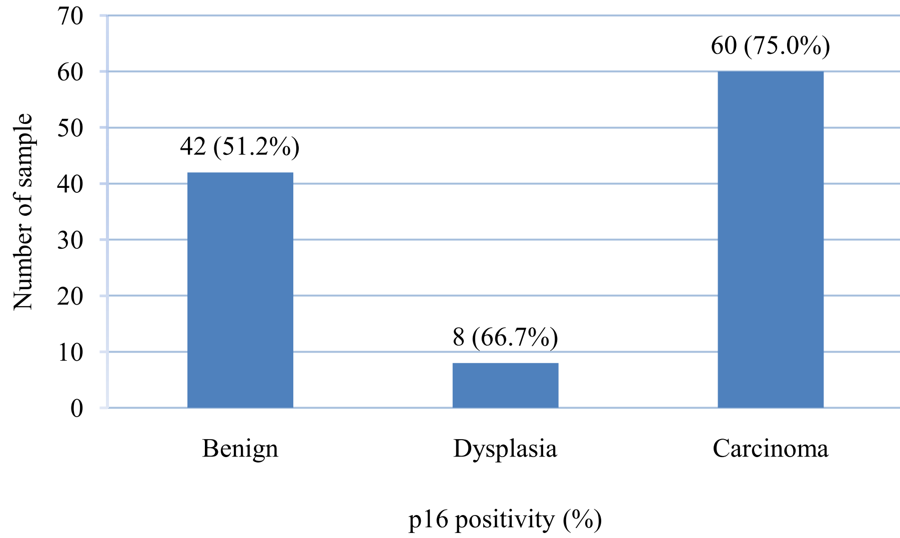



**Figure 5.2: Overall p16 status in each pathological group**

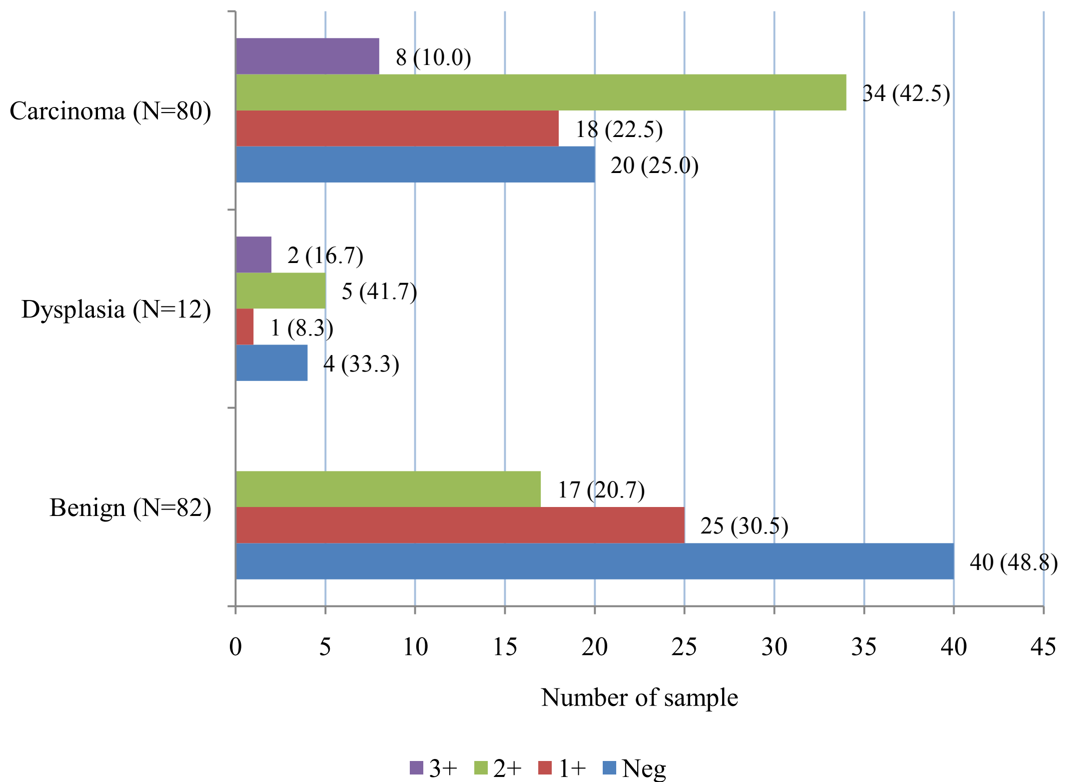



**Figure 5.3: The frequency of p16 staining intensity in each pathological group.** N = number of samples; the frequency and the percentage (%)

### 5.3.1 A comparison of p16 expression in benign, dysplasia and carcinoma groups

In this cohort, it was demonstrated that 110/174 cases (63.2%) were positive for p16 staining and the rest, 64/174 cases (36.8%) were negative, regardless of their pathological groups. Figure 5.2 shows the positive expression of p16, 42/82 (51.2%) in benign, 8/12 (66.7%) in dysplasia and 60/80 (75.0%) in carcinoma samples. The Kruskal-Wallis test indicates that p16 positivity significantly differs across the three pathological groups ($p < 0.01$) [Table 5.2 (b)]. The Mann-Whitney U test was carried out separately, to compare p16 positivity between benign versus dysplasia groups, benign versus carcinoma groups and dysplasia versus carcinoma groups. We found that p16 positivity in the carcinoma group was significantly higher compared to benign group ($p < 0.01$) [Table 5.2 (d)]. Whereas, p16 positive between carcinoma and dysplasia groups ($p = 0.542$) or between dysplasia and benign groups ($p = 0.319$) was comparable.

We observed the proportion of p16 staining intensity (2+ score) in carcinoma was 34/80 (42.5%), more than double that shown in benign group, 17/82 (20.7%). The final ratio of 3+ score of the former group was 4:1 compared to dysplasia group. The former group, on the contrary, exhibited more than fifty percent less negative p16 staining compared to the latter group. Similarly, there was a significantly different in overall p16 staining intensity across pathological group ($p < 0.001$) [Table 5.3 (b)]. We revealed that overall p16 staining intensity in carcinoma group was significantly higher compared to benign group ($p < 0.001$) [Table 5.3 (d)]. In addition, p16 staining intensity in dysplasia group was significantly higher compared to benign group ($p = 0.036$).

There were no significant different between carcinoma and dysplasia groups ($p$ = 0.845).

**Table 5.2: Kruskal-Wallis and Mann-Whitney tests on p16 positivity**

(a)

**Ranks**

| Pathological group | | N | Mean Rank |
|---|---|---|---|
| p16 positivity | Benign | 82 | 77.06 |
| | Dysplasia | 12 | 90.50 |
| | Carcinoma | 80 | 97.75 |
| | Total | 174 | |

(b)

**Test Statistics[a,b]**

| | p16 positivity |
|---|---|
| Chi-Square | 9.857 |
| *df* | 2 |
| Asymp. Sig. | .007 |

a. Kruskal Wallis Test

b. Grouping Variable: Pathological group

(c)

**Ranks**

| Pathological group | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| p16 positivity | Benign | 82 | 71.99 | 5903.00 |
| | Carcinoma | 80 | 91.25 | 7300.00 |
| | Total | 162 | | |

(d)

**Test Statistics[a]**

| | p16 status |
|---|---|
| Mann-Whitney U | 2500.000 |
| Wilcoxon W | 5903.000 |
| Z | -3.124 |
| Asymp. Sig. (2-tailed) | .002 |

a. Grouping Variable: Pathological group

**Table 5.3: Kruskal-Wallis and Mann-Whitney tests on p16 staining intensity**

(a)

**Ranks**

| Pathological group | | N | Mean Rank |
|---|---|---|---|
| p16 intensity | Benign | 82 | 70.52 |
| | Dysplasia | 12 | 103.17 |
| | Carcinoma | 80 | 102.55 |
| | Total | 174 | |

(b)

**Test Statistics[a,b]**

| | p16 intensity |
|---|---|
| Chi-Square | 19.559 |
| *df* | 2 |
| Asymp. Sig. | .000 |

a. Kruskal Wallis Test

b. Grouping Variable: Pathological group

(c)

**Ranks**

| Pathological group | | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| p16 intensity | Benign | 82 | 66.62 | 5463.00 |
| | Carcinoma | 80 | 96.75 | 7740.00 |
| | Total | 162 | | |

(d)

**Test Statistics[a]**

| | p16 intensity |
|---|---|
| Mann-Whitney U | 2060.000 |
| Wilcoxon W | 5463.000 |
| Z | -4.310 |
| Asymp. Sig. (2-tailed) | .000 |

a. Grouping Variable: Pathological group

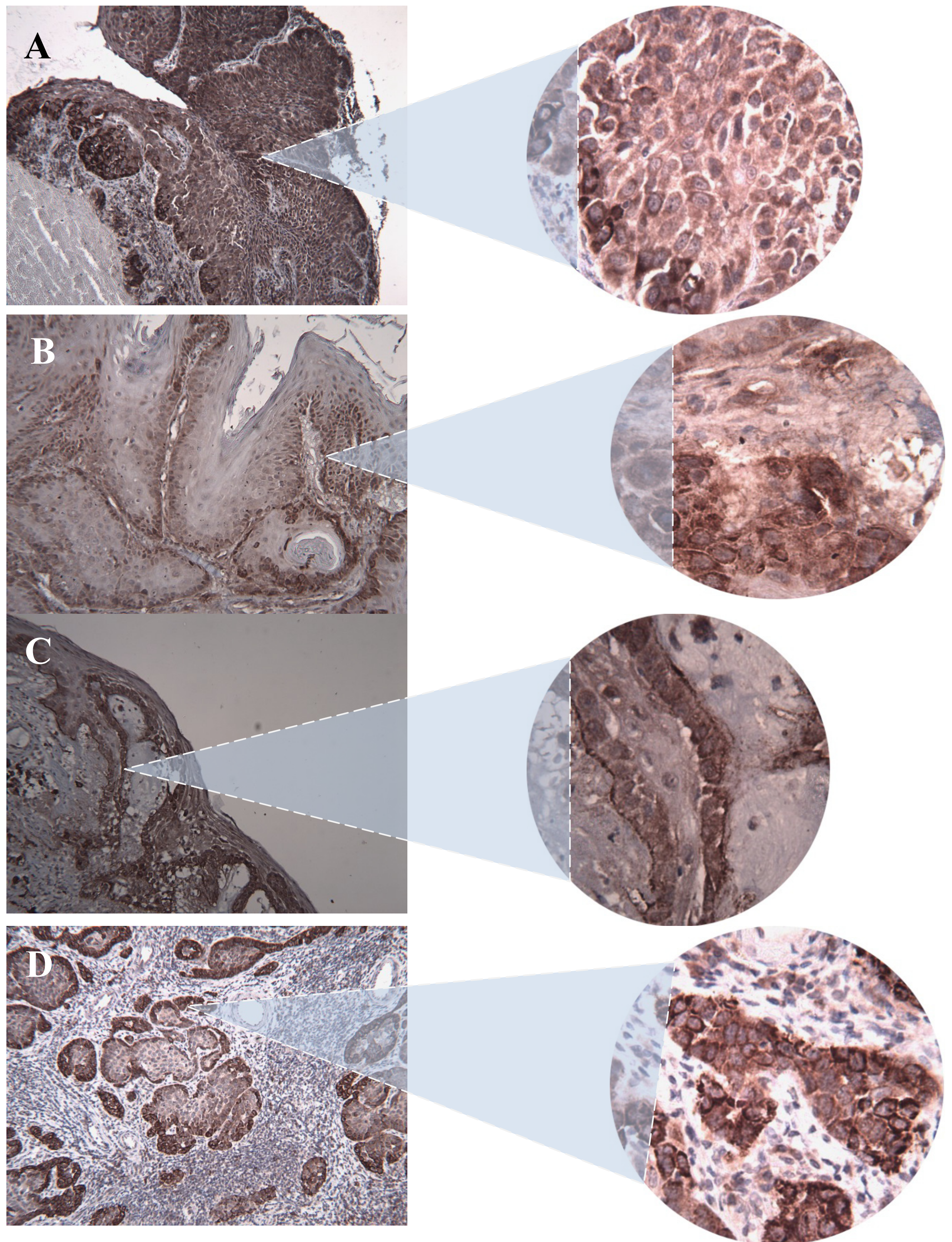



**Figure 5.4: Photomicrograph of p16 staining across histological grade.**
(A) Well differentiated (B) Moderately differentiated (C) Basaloid and (D) Poorly differentiated at x100 magnification (rectangular) and x400 magnification (circle)

### 5.3.2 A comparison of p16 status according to demographic and clinical parameters in each pathological group

The Chi-squared, the Kruskal-Wallis H and the Mann-Whitney U tests were utilised for this comparison where appropriate. In benign and dysplasia groups, no significant difference was observed in both p16 positivity and p16 staining intensity across the following parameters, gender, age group, anatomic sites, diseases status and disease outcome (Table 5.4). Similar results were observed in both pathological groups in relation to p16 staining intensity (Table 5.5).

Unlike the first 2 groups, in carcinoma group, significantly higher p16 positivity and p16 staining intensity was found in males compared to their counterparts ($p<0.01$). Younger patients, on the other hand, demonstrated significantly less p16 positivity and staining intensity compared to the older ones ($p<0.001$). Overall comparison of p16 status resulted in a significant difference based on anatomic sites at $p<0.001$ in both parameters (p16 positivity and p16 staining intensity). Among seven anatomic sites, we regrouped them into two distinct groups based on the overall percentage of p16 positivity and p16 staining intensity. The first (with lower percentage of p16 positivity and p16 staining intensity) consisted of buccal mucosa, gingivae, retromolar pad and lower lip. The members of the second group (with higher percentage of p16 positivity and p16 staining intensity) were floor of the mouth, soft palate and the tongue. No significant difference was observed in both parameters compared to the first group. p16 positivity and p16 staining intensity were demonstrated to be significantly different in the second group. Both parameters were significantly higher in the tongue compared to soft palate [($p<0.01$) and ($p<0.001$) for p16 positivity and p16 staining intensity,

respectively]. Similarly, p16 positivity and p16 staining intensity were higher in floor of the mouth compared to soft palate at ($p<0.05$) and ($p<0.01$), respectively. The tongue and floor of the mouth appeared to be comparable for p16 status [($p=0.716$) and ($p=0.158$)]. In addition, higher p16 positivity and p16 staining intensity observed in deceased patients at $p<0.001$ in both parameters.

## Table 5.4: A comparison of p16 positivity in each pathological group

| Parameter | *N* | Benign (*N* = 82) | | | | Dysplasia (*N* = 12) | | | | Carcinoma (*N* = 80) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | p16- (%) | p16+ (%) | $\chi^2$ | *p* value | p16- (%) | p16+ (%) | $\chi^2$ | *p* value | p16- (%) | p16+ (%) | $\chi^2$ | *p* value |
| Gender | | | | | | | | | | | | | |
| Female | 97 | 19 (47.5) | 24 (57.1) | 0.76 | 0.382 | 3 (75.0) | 2 (25.0) | 2.74 | 0.098 | 19 (95.0) | 30 (50.0) | 12.80** | 0.000 |
| Male | 77 | 21 (52.5) | 18 (42.9) | | | 1 (25.0) | 6 (75.0) | | | 1 (5.0) | 30 (50.0) | | |
| Age group | | | | | | | | | | | | | |
| 16-57y | 90 | 25 (62.5) | 25 (59.5) | 0.08 | 0.782 | - | 2 (25.0) | 1.20 | 0.273 | 18 (90.0) | 20 (33.3) | 19.32** | 0.000 |
| 58-99y | 84 | 15 (37.5) | 17 (40.5) | | | 4 (100.0) | 6 (75.0) | | | 2 (10.0) | 40 (66.7) | | |
| Anatomic site | | | | | | | | | | | | | |
| Buccal mucosa | 45 | 17 (42.5) | 24 (57.1) | 6.02 | 0.738 | - | 1 (12.5) | 3.56 | 0.313 | - | 3 (5.0) | 25.94** | 0.000 |
| Floor of the mouth | 23 | 1 (2.5) | 2 (4.8) | | | 3 (75.0) | 5 (62.5) | | | 1 (5.0) | 11 (18.3) | | |
| Gingivae | 9 | 3 (7.5) | 2 (4.8) | | | - | - | | | 1 (5.0) | 3 (5.0) | | |
| Hard palate | 3 | 2 (5.0) | - | | | 1 (25.0) | - | | | - | - | | |
| Soft palate | 39 | 2 (5.0) | 1 (2.4) | | | - | 2 (25.0) | | | 18 (90.0) | 16 (26.7) | | |
| Upper lip | 2 | 1 (2.5) | 1 (2.4) | | | - | - | | | - | - | | |
| Lower lip | 8 | 2 (5.0) | 2 (4.8) | | | - | - | | | - | 4 (6.7) | | |
| Retromolar pad | 4 | 1 (2.5) | - | | | - | - | | | - | 3 (5.0) | | |
| Skin | 1 | 1 (2.5) | - | | | - | - | | | - | - | | |
| Tongue | 40 | 10 (25.0) | 10 (23.8) | | | - | - | | | - | 20 (33.3) | | |
| | | | - | | | | | | | | | | |
| Disease status | | | | | | | | | | | | | |
| No recurrence | 169 | 40 (100.0) | 42 (100.0) | N/A | N/A | 4 (100.0) | 8 (100.0) | N/A | N/A | 20 (100) | 55 (91.7) | 1.78 | 0.182 |
| Recurrence | 5 | - | - | | | - | - | | | - | 2 (8.3) | | |
| Disease outcome | | | | | | | | | | | | | |
| Alive | 45 | - | - | N/A | N/A | - | - | N/A | N/A | 19 (95.0) | 26 (43.3) | 16.27** | 0.000 |
| Dead | 35 | - | - | | | - | - | | | 1 (5.0) | 34 (56.7) | | |

N/A; not available; ** Statistically significant at the 0.01 level and * at the 0.05 level

## Table 5.5: A comparison of p16 staining intensity in each pathological group

| Parameter | *N* | Benign (*N* = 82) | | | | | | Dysplasia (*N* = 12) | | | | | | Carcinoma (*N* = 80) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Neg (%) | 1+ (%) | 2+ (%) | 3+ (%) | $\chi^2$ | *p* value | Neg (%) | 1+ (%) | 2+ (%) | 3+ (%) | $\chi^2$ | *p* value | Neg (%) | 1+ (%) | 2+ (%) | 3+ (%) | $\chi^2$ | *p* value |
| Gender | | | | | | | | | | | | | | | | | | | |
| Female | 97 | 19 (47) | 14 (56) | 10 (59) | - | 0.80 | 0.672 | 3 (75) | - | 1 (20) | 1 (50) | 3.57 | 0.312 | 19 (95) | 11 (61) | 17 (50) | 2 (25) | 15.84** | 0.001 |
| Male | 77 | 21 (53) | 11 (44) | 7 (41) | - | | | 1 (25) | 1 (100) | 4 (80) | 1 (50) | | | 1 (5) | 7 (39) | 17 (50) | 6 (75) | | |
| Age group | | | | | | | | | | | | | | | | | | | |
| 16-57y | 90 | 25 (63) | 14 (56) | 11 (65) | - | 0.40 | 0.819 | - | 1 (100) | 1 (20) | - | 6.24 | 0.100 | 18 (90) | 9 (50) | 9 (26) | 2 (25) | 22.19** | 0.000 |
| 58-99y | 84 | 15 (37) | 11 (44) | 6 (35) | - | | | 4 (100) | - | 4 (80) | 2 (100) | | | 2 (10) | 9 (50) | 25 (74) | 6 (75) | | |
| Anatomic site | | | | | | | | | | | | | | | | | | | |
| Buccal mucosa | 45 | 17 (42) | 15 (60) | 9 (53) | - | 12.03 | 0.845 | - | - | 1 (20) | - | 5.93 | 0.747 | - | - | 3 (9) | - | 41.57** | 0.001 |
| Floor of the mouth | 23 | 1 (2) | 2 (8) | - | - | | | 3 (75) | 1 (100) | 3 (60) | 1 (50) | | | 1 (5) | 3 (17) | 8 (24) | - | | |
| Gingivae | 9 | 3 (7) | 1 (7) | 1 (6) | - | | | - | - | - | - | | | 1 (5) | 1 (6) | 2 (6) | - | | |
| Hard palate | 3 | 2 (5) | - | - | - | | | 1 (25) | - | - | - | | | - | - | - | - | | |
| Soft palate | 39 | 2 (5) | - | 1 (6) | - | | | - | - | 1 (20) | 1 (50) | | | 18 (90) | 7 (21) | 7 (21) | 2 (25) | | |
| Upper lip | 2 | 1 (2) | - | 1 (6) | - | | | - | - | - | - | | | - | - | - | - | | |
| Lower lip | 8 | 2 (5) | 2 (8) | - | - | | | - | - | - | - | | | - | 2 (11) | 2 (6) | - | | |
| Retromolar pad | 4 | 1 (2) | - | - | - | | | - | - | - | - | | | - | 1 (6) | 2 (6) | - | | |
| Skin | 1 | 1 (2) | - | - | - | | | - | - | - | - | | | - | - | - | - | | |
| Tongue | 40 | 10 (25) | 5 (20) | 5 (29) | - | | | - | - | - | - | | | - | 4 (22) | 10 (29) | 6 (75) | | |
| Disease status | | | | | | | | | | | | | | | | | | | |
| No recurrence | 169 | 40 (100) | 25 (100) | 17 (100) | - | N/A | N/A | 4 (100) | 1 (100) | 5 (100) | 2 (100) | N/A | N/A | 20 (100) | 18 (100) | 30 (88) | 7 (87) | 4.83 | 0.185 |
| Recurrence | 5 | - | - | - | - | | | - | - | - | - | | | - | - | 4 (12) | 1 (13) | | |
| Disease outcome | | | | | | | | | | | | | | | | | | | |
| Alive | 45 | - | - | - | - | N/A | N/A | - | - | - | - | N/A | N/A | 1 (5) | 7 (39) | 21 (62) | 6 (75) | 20.03** | 0.000 |
| Dead | 35 | - | - | - | - | | | - | - | - | - | | | 19 (95) | 11 (61) | 13 (38) | 2 (25) | | |

N/A - not available; ** Statistical significant at the 0.01 level and * at the 0.05 level

## Table 5.6: A comparison of p16 positivity in carcinoma group only

| Parameter | $N$ | Carcinoma ($N$ = 80) | | | |
|---|---|---|---|---|---|
| | | p16- (%) | p16+ (%) | $\chi^2$ | $p$ value |
| Tumour size | | | | | |
| $T_1$ (<2 cm) | 41 | 20 (100.0) | 21 (35.5) | 25.37** | 0.000 |
| $T_2$ (2-4 cm) | 22 | 0 (0) | 22 (36.7) | | |
| $T_3$ (>4 cm) | 13 | 0 (0) | 13 (21.7) | | |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | 0 (0) | 4 (6.7) | | |
| Regional Lymph Nodes | | | | | |
| $N_0$ | 67 | 19 (95.0) | 48 (80.0) | 2.96 | 0.228 |
| $N_1$ | 7 | 0 (0) | 7 (11.7) | | |
| $N_2$ | 6 | 1 (5.0) | 5 (8.3) | | |
| Histological grade | | | | | |
| Well differentiated | 9 | 0 (0) | 9 (15.0) | 9.20* | 0.027 |
| Moderately differentiated | 58 | 19 (95.0) | 39 (65.0) | | |
| Basaloid | 2 | 1 (5.0) | 1 (1.7) | | |
| Poorly differentiated | 11 | 0 (0) | 11 (18.3) | | |

N/A - not available; ** Statistical significant at the 0.01 level and * at the 0.05 level

## Table 5.7: A comparison of p16 staining intensity in carcinoma group only

| Parameter | $N$ | Carcinoma ($N$ = 80) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Neg (%) | 1+ (%) | 2+ (%) | 3+ (%) | $\chi^2$ | $p$ value |
| Tumour size | | | | | | | |
| $T_1$ (<2 cm) | 41 | 20 (100.0) | 11 (61.1) | 9 (26.5) | 1 (12.5) | 40.62** | 0.000 |
| $T_2$ (2-4 cm) | 22 | - | 5 (27.8) | 12 (35.3) | 5 (62.5) | | |
| $T_3$ (>4 cm) | 13 | - | - | 11 (32.4) | 2 (25.0) | | |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | - | 2 (11.1) | 2 (5.9) | - | | |
| Regional Lymph Nodes | | | | | | | |
| $N_0$ | 67 | 19 (95.0) | 14 (77.8) | 26 (76.5) | 8 (100.0) | 5.95 | 0.429 |
| $N_1$ | 7 | - | 2 (11.1) | 5 (14.7) | - | | |
| $N_2$ | 6 | 1 (5.0) | 2 (11.1) | 3 (8.8) | - | | |
| Histological grade | | | | | | | |
| Well differentiated | 9 | - | 4 (22.2) | 4 (11.8) | 9 (15.0) | 14.15 | 0.117 |
| Moderately differentiated | 58 | 19 (95.0) | 13 (72.2) | 21 (61.8) | 5 (62.5) | | |
| Basaloid | 2 | 1 (5.0) | - | 1 (2.9) | - | | |
| Poorly differentiated | 11 | - | 1 (5.6) | 8 (23.5) | 2 (25.0) | | |

N/A - not available; ** Statistical significant at the 0.01 level and * at the 0.05 level

p16 positivity (Table 5.6) and p16 staining intensity (Table 5.7) were significantly different across tumour size in carcinoma group ($\chi^2$=25.4, $p$<0.001) and ($\chi^2$=40.6, $p$<0.001), respectively. Within tumour size comparisons were made and we observed $T_3$ and $T_2$ tumour size, each were significantly higher compared to $T_1$ for p16 status [each at $p$<0.01 and $p$<0.001 for p16 positivity and p16 staining intensity, respectively]. Comparison amongst $T_2$, $T_3$ and $T_4$ for p16 status appeared to be comparable. No significant difference was observed for p16 status in the regional lymph nodes and similarly for p16 staining intensity in histological grade. This was not the case for p16 positivity where overall histological grade was significantly different at $p$<0.05. p16 positivity in well and poorly differentiated tissues were significantly higher over moderately differentiated tissues at p=0.044 and p=0.027, respectively.

## 5.4 THE RISK FACTORS AMONG DEMOGRAPHIC AND CLINICAL PARAMETERS BY p16 STATUS

The analysis of demographic and clinical (Table 5.8) and histopathological risk factor characteristics (Table 5.9) of oral cancer in each pathological group by p16 status were carried out. The data on histopathological risk factor characteristics were available only for carcinoma group for this study. In carcinoma patients, the odds ratio was statistically significant different for disease recurrence [OR=2.7, 95%CI (1.6-4.6), $p$<0.001] and regional lymph nodes [OR=2.5, 95%CI (1.5-4.3), $p$<0.01] by p16 status compared to reference group.

## Table 5.8: Demographic and risk factor characteristics by p16 status in each pathological group

| Parameter | *N* | Benign (*N* = 82) | | | | Dysplasia (*N* = 12) | | | | Carcinoma (*N* = 80) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | p16- (%) | p16+ (%) | OR (95%CI) | *p* value | p16- (%) | p16+ (%) | OR (95%CI) | *p* value | p16- (%) | p16+ (%) | OR (95%CI) | *p* value |
| Gender | | | | | | | | | | | | | |
| Female[α] | 97 | 19 (47.5) | 24 (57.1) | 1.0 | 0.383 | 3 (75.0) | 2 (25.0) | 1.0 | 0.657 | 19 (95.0) | 30 (50.0) | 1.0 | 0.119 |
| Male | 77 | 21 (52.5) | 18 (42.9) | 0.7 (0.3-1.6) | | 1 (25.0) | 6 (75.0) | 0.7 (0.1-4.0) | | 1 (5.0) | 30 (50.0) | 1.6 (0.9-2.8) | |
| Age group | | | | | | | | | | | | | |
| 16-57y[α] | 90 | 25 (62.5) | 25 (59.5) | 1.0 | 0.782 | - | 2 (25.0) | N/E | 0.999 | 18 (90.0) | 20 (33.3) | 1.0 | 0.746 |
| 58-99y | 84 | 15 (37.5) | 17 (40.5) | 1.1 (0.5-2.8) | | 4 (100.0) | 6 (75.0) | | | 2 (10.0) | 40 (66.7) | 1.1 (0.6-2.1) | |
| Anatomic site | | | | | | | | | | | | | |
| Buccal mucosa[α] | 45 | 17 (42.5) | 24 (57.1) | 1.0 | 0.997 | - | 1 (12.5) | 1.0 | 0.921 | - | 3 (5.0) | 1.0 | 0.391 |
| Floor of the mouth | 23 | 1 (2.5) | 2 (4.8) | 1.4 (0.1-16.9) | 0.783 | 3 (75.0) | 5 (62.5) | N/E | | 1 (5.0) | 11 (18.3) | N/E | 0.999 |
| Gingivae | 9 | 3 (7.5) | 2 (4.8) | 0.5 (0.1-3.1) | 0.437 | - | - | | | 1 (5.0) | 3 (5.0) | 11 (1.4-85.2) | 0.022 |
| Hard palate | 3 | 2 (5.0) | - | N/E | 0.999 | 1 (25.0) | - | 1.7 (0.4-7.0) | 0.484 | - | - | | |
| Soft palate | 39 | 2 (5.0) | 1 (2.4) | 0.4 (0.0-4.2) | 0.412 | - | 2 (25.0) | N/E | | 18 (90.0) | 16 (26.7) | 3.0 (0.3-28.8) | 0.341 |
| Upper lip | 2 | 1 (2.5) | 1 (2.4) | 0.7 (0.0-12.1) | 0.812 | - | - | | | - | - | | |
| Lower lip | 8 | 2 (5.0) | 2 (4.8) | 0.7 (0.1-5.5) | 0.742 | - | - | | | - | - | 0.9 (0.5-1.7) | 0.732 |
| Retromolar pad | 4 | 1 (2.5) | - | N/E | 1.000 | - | - | | | - | 4 (6.7) | N/E | 0.999 |
| Skin | 1 | 1 (2.5) | - | N/E | 1.000 | - | - | | | - | 3 (5.0) | | |
| Tongue | 40 | 10 (25.0) | 10 (23.8) | 0.7 (0.2-2.1) | 0.529 | - | - | | | - | 20 (33.3) | N/E | 0.999 |
| | | | - | | | | | | | | | | |
| Disease status | | | | | | | | | | | | | |
| No recurrence[α] | 169 | 40 (100.0) | 42 (100.0) | N/A | N/A | 4 (100.0) | 8 (100.0) | N/A | N/A | 20 (100) | 55 (91.7) | 1.0 | 0.000 |
| Recurrence | 5 | - | - | | | 0 (0) | - | | | - | 2 (8.3) | 2.7 (1.6-4.6) | |
| Disease outcome | | | | | | | | | | | | | |
| Alive | 45 | - | - | | | - | - | N/A | N/A | 19 (95.0) | 26 (43.3) | 1.4 (0.8-2.5) | 0.299 |
| Dead[α] | 35 | - | - | N/A | N/A | - | - | | | 1 (5.0) | 34 (56.7) | 1.0 | |

[α] – reference group; N/A; not available; N/E – not estimable

**Table 5.9: Histopathological risk factor properties by p16 status in carcinoma group**

| Parameter | $N$ | Carcinoma ($N$ = 80) | | | |
|---|---|---|---|---|---|
| | | p16- (%) | p16+ (%) | OR (95%CI) | $p$ value |
| Tumour size | | | | | |
| $T_1$ (<2 cm)$^\alpha$ | 41 | 20 (100.0) | 21 (35.5) | 1.0 | 0.999 |
| $T_2$ (2-4 cm) | 22 | - | 22 (36.7) | 1.1 (0.6-1.9) | 0.876 |
| $T_3$ (>4 cm) | 13 | - | 13 (21.7) | N/E | 0.998 |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | - | 4 (6.7) | N/E | 0.998 |
| Regional Lymph Nodes | | | | | |
| $N_0$$^\alpha$ | 67 | 19 (95.0) | 48 (80.0) | 1.0 | 0.003 |
| $N_1$ | 7 | - | 7 (11.7) | 2.5 (1.5-4.3) | 0.001 |
| $N_2$ | 6 | 1 (5.0) | 5 (8.3) | N/E | 0.999 |
| Histological grade | | | | | |
| Well differentiated$^\alpha$ | 9 | - | 9 (15.0) | 1.0 | 0.010 |
| Moderately differentiated | 58 | 19 (95.0) | 39 (65.0) | N/E | 0.999 |
| Poorly differentiated[1] | 11 | - | 11 (18.3) | 2.1 (0.12-34.6) | 0.618 |
| Basaloid | 2 | 1 (5.0) | 1 (1.7) | N/E | 0.999 |

$^\alpha$ – reference group; N/E – not estimable

## 5.5 CORRELATION BETWEEN DEMOGRAPHIC/CLINICOPATHOLOGICAL PARAMETERS AND p16 STATUS

The correlation analysis was undertaken searching for any association between clinical parameters in benign, dysplasia and carcinoma with p16 positivity (Appendix 51) and p16 staining intensity (Appendix 52). No association was observed in all demographic and clinical parameters with p16 status in benign and dysplasia groups.

p16 positivity was associated with gender ($r$=0.400, $p$<0.001), age group ($r$=0.491, $p$<0.001), tumour size ($r$=0.416, $p$<0.001) but exhibited negative association for disease status ($r$=-0.451, $p$<0.001) in carcinoma patients. p16 staining intensity demonstrated positive correlation with gender ($r$=0.359, $p$<0.01), age group ($r$=0.524, $p$<0.001), anatomic site ($r$=0.242, $p$<0.001), disease outcome ($rho$=0.222, $p$<0.05),

$p<0.001$), tumour size ($r=0.471$, $p<0.001$) and was negatively associated in disease status ($r=-0.448$, $p<0.001$).

## 5.6 SURVIVAL ANALYSIS

This analysis is only applicable for the carcinoma group, in which relying upon the data of patient survival (alive/dead) and the sample size is 80.

### 5.6.1 Kaplan-Meier Survival Analysis

The results of this analysis suggested that the overall patient survival was worse in one group (Table 5.8):

1. p16 positive patients compared with the negative ones ($p<0.05$) (Figure 5.5).

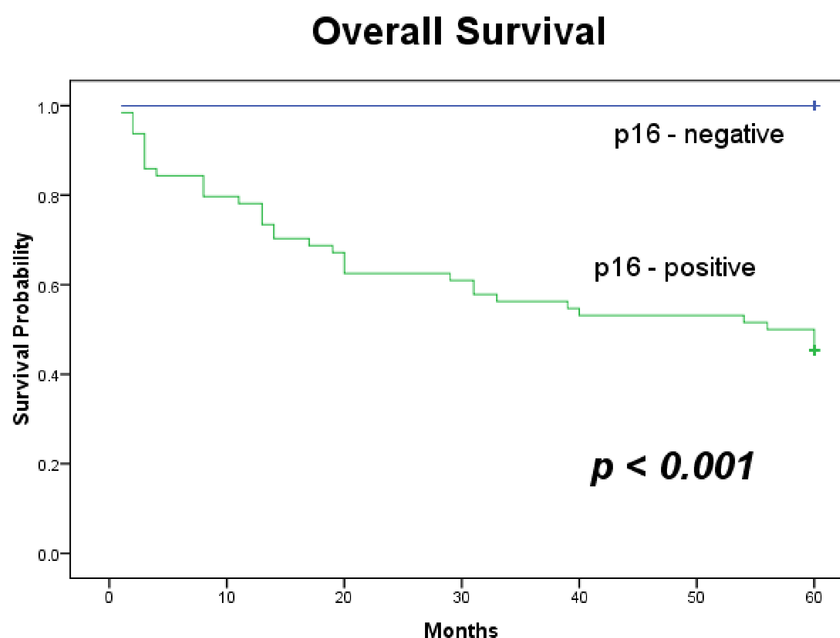



**Figure 5.5: An association between p16 positivity and overall survival as generated by Kaplan-Meier test.** Vertical tick marks on curves indicate censored observations.

**Table 5.10: The prognostic significance from overall survival analysis in carcinoma patients**

| Parameter (*N*=80) | Log Rank (Mantel-Cox) | | |
|---|---|---|---|
| | *Chi-Square* | *df* | *Sig.* |
| p16 positivity | 12.568 | 1 | 0.000** |
| p16 staining intensity | 16.192 | 3 | 0.001** |
| p16/HPV status | 13.433 | 3 | 0.004** |
| p16/α-HPV status | 13.660 | 3 | 0.003** |
| p16/HR-HPV status | 13.494 | 3 | 0.004** |
| p16/LR-HPV status | 15.663 | 3 | 0.001** |
| p16/HPV16 status | 16.009 | 3 | 0.001** |
| p16/other HPV types | 12.603 | 3 | 0.006** |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

### 5.6.2 Cox Regression Model

Multifactorial effect on patient survival as analysed by Cox regression model. These prognostic factors exhibited independently disagreement with overall survival analysis by Kaplan-Meier test except for p16 positivity (Table 5.11).

**Table 5.11: The summary of the survival analysis done by Cox regression**

| Parameter (*N*=80) | Cox Regression Analysis | | |
|---|---|---|---|
| | *Wald* | *df* | *Sig.* |
| p16 positivity | 4.483 | 1 | 0.034* |
| p16 staining intensity | 2.577 | 3 | 0.462 |
| p16/HPV status | 0.666 | 3 | 0.881 |
| p16/α-HPV status | 0.833 | 3 | 0.841 |
| p16/HR-HPV status | 0.670 | 3 | 0.880 |
| p16/LR-HPV status | 2.249 | 3 | 0.522 |
| p16/HPV16 status | 5.417 | 3 | 0.144 |
| p16/Other HPV types | 0.033 | 3 | 0.998 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

## 5.7 CONCLUSIONS

Our results thus far for this cohort could conclude:

1. **p16 expression in benign, dysplasia and carcinoma groups**

    - The percentage of p16 expression are as follow, 42/82 (51.2%) in benign, 8/12 (66.7%) in dysplasia and 60/80 (75.0%) in carcinoma.
    - p16 positivity and p16 staining intensity were significantly higher in carcinoma compared to benign with ($p<0.01$) for both but were comparable between carcinoma-dysplasia and dysplasia-benign.

2. **A comparison of p16 expression based on demographic and clinicpathological parameters in benign, dysplasia and carcinoma groups**

    - No significant difference was observed for p16 status (positivity and staining intensity) in benign and dysplasia groups based on the following demographic and clinical parameters, gender, age group, anatomic site, disease status and disease outcome.
    - Carcinoma group, on the other hand, demonstrated significantly higher p16 status for male, older patients, deceased patients compared to their respective counterparts and anatomic site in the following order (tongue > soft palate or floor of the mouth > soft palate).
    - In the carcinoma group, p16 status was significantly higher each in $T_2$ and $T_3$ compared with $T_1$ but p16 status were comparable amongst $T_2$, $T_3$ and $T_4$.
    - No significant different in p16 status within regional lymph nodes in carcinoma group.

- In carcinoma group, p16 positivity was significantly higher in well and poorly differentiated tissues where each was compared with moderately differentiated tissues.
- Two cases were identified in carcinoma where significant estimate for odds ratio observed by p16 status i.e. disease recurrence and $N_1$ compared to each reference group.

**3. Correlation between demographic/clinicopathological parameters and p16 status**

- Neither p16 positivity nor p16 staining intensity demonstrated any correlation with demographic and clinicopathological parameters in dysplasia patients.
- In benign, a negative association was observed both for p16 positivity and p16 staining intensity with anatomic site.
- Generally, most of the association between p16 status and demographic/ clinicopathological parameters was observed in the carcinoma group.
- Similarity in both parameters where positive associations were identified in gender, age group, tumour size and negatively associated in disease outcome.
- In addition, p16 positivity in carcinoma was positively correlated with anatomic site and disease status.
- At this stage, we could deduce the potential of p16 as an indicator for oral disease progression in parallel to HPV, as it follows the similar pattern as HPVs.

**4. Potential association with survival for p16**

- The survival of the patients was worst in positively identified p16 status compared to the negative ones based on Kaplan-Meier test and this was confirmed by Cox regression analysis.

# CHAPTER 6

# CORRELATION BETWEEN HPVs AND p16 EXPRESSION IN HEAD AND NECK CANCER

## 6.1 OVERVIEW

An overview of the aims of this chapter is given as follows:

- To analyse the pooled data of 140 head and neck carcinomas (60 FFPETs from the pilot study and 80 FFPETs from the oral disease progression study).
- To determine whether there is any association between HPV prevalence and p16 status with demographic and clinicopathological parameters.
- To determine whether there is an association between HPV and p16 in head and neck carcinomas.

## 6.2 CLINICAL DATA

The cohort included 140 patients with primarily squamous cell carcinoma and a small minority with carcinoma in situ and severe dysplasia. The male:female ratio was 16:19 with the mean age of 67.5 years, ranging from 36-99 years. An average age was 67.2 and 59.8 for male and female, respectively. As for the tumour sites, 49 cases (35%) were on the tongue; soft palate, 37 cases (26.4%); floor of the mouth, 22 cases (15.7%); retromolar pad, 12 cases (8.6%); lower lip, 5 cases (3.6%); four cases each (2.9%) for buccal mucosa, alveolus and gingivae and one case each (0.7%) for pharynx,

supraglottis and tonsil. The clinicopathological parameters recorded in each tumour consist of the tumour size and regional lymph node as shown in Table 6.1.

**Table 6.1: Clinical details of oral cancer patients**

| Parameters | *N* | % |
|---|---|---|
| Total | 140 | |
| Gender | | |
| Female | 76 | 54.3 |
| Male | 64 | 45.7 |
| Age group[α] (range, 36-99y) | | |
| 36-64y | 75 | 53.6 |
| 65-99y | 65 | 46.4 |
| Anatomic site | | |
| Buccal mucosa | 4 | 2.9 |
| Floor of the mouth | 22 | 15.7 |
| Alveolus | 4 | 2.9 |
| Gingivae | 4 | 2.9 |
| Soft palate | 37 | 26.4 |
| Pharynx | 1 | 0.7 |
| Lower lip | 5 | 3.6 |
| Retromolar pad | 12 | 8.6 |
| Supraglottis | 1 | 0.7 |
| Tongue | 49 | 35.0 |
| Tonsil | 1 | 0.7 |
| Tumour size | | |
| Negative | 2 | 1.4 |
| $T_1$ (<2 cm) | 65 | 46.4 |
| $T_2$ (2-4 cm) | 41 | 29.3 |
| $T_3$ (>4 cm) | 14 | 10.0 |
| $T_4$ (>4 cm) and invades adjacent structures | 18 | 12.9 |
| Regional lymph nodes | | |
| Negative | 71 | 50.7 |
| $N_0$ | 35 | 25.0 |
| $N_1$ | 16 | 11.4 |
| $N_2$ | 18 | 12.9 |
| Disease status | | |
| No recurrence | 129 | 92.1 |
| Recurrence | 11 | 7.9 |
| Disease outcome | | |
| Alive | 81 | 57.9 |
| Dead | 59 | 42.1 |

*Abbreviation: N, number of cases;* [α] *It was based on median age*

## 6.3 HPVs EXPRESSION IN ORAL MUCOSA

The conventional PCR (semi-nested and nested PCR) was utilised for α-HPV detection in both cohorts. Therefore, the results of similar techniques from the pilot study were included in the pooled data of 140 carcinomas. We have to note that the detection of β-HPV types was not investigated in the larger sample of 80 carcinomas. Therefore, the data from the pilot study involving β-HPV types was excluded in the pooled data of 140 carcinomas and we focused our discussion more on HR-HPV and LR-HPV. Table 6.2 and Figure 6.1 show the positively detected HPV types in this cohort. In total 82/140 cases (58.6%) were positive for HPV regardless the type of HPV. The highest proportion was led by LR α-HPV types, 58/140 (41.4%) followed by HR α-HPV types, 24/140 (17.1%), and other HPV types 16/140 (11.4%). Figure 6.2 and 6.3 illustrate the distribution of positively detected HPV types.

**Table 6.2: Overall HPV types detected in oral carcinoma (*N*=140)**

| HPV group | HPV type | HPV type *N* (%) | HPV group *N* (%) |
|---|---|---|---|
| Low risk α-HPV | HPV6 | 58 (41.4) | 58 (41.4) |
| High risk α-HPV | HPV16 | 17 (12.1) | 24 (17.1) |
| | HPV18 | 4 (2.9) | |
| | HPV35 | 1 (0.7) | |
| | Untypable | 2 (1.4) | |
| Other HPV types | HPV64 | 1 (0.7) | 16 (11.4) |
| | HPV100 | 2 (1.4) | |
| | HPV103 | 1 (0.7) | |
| | HPV109 | 1 (0.7) | |
| | β-HPV from pilot study | 11 (7.9) | |
| | Total LR-HPV and HR-HPV(positive) | 82 | |
| | Percentage | 58.6 | |
| | Total sample | 140 | |

*N – number of sample; The percentage was calculated based on the total number of sample, 140*

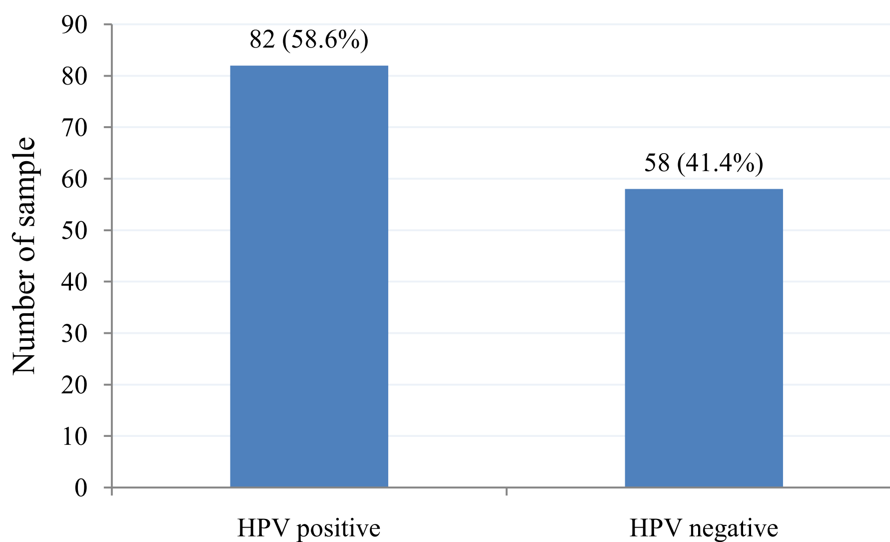



**Figure 6.1: The total of HPV positively detected in the pooled data of oral carcinomas**

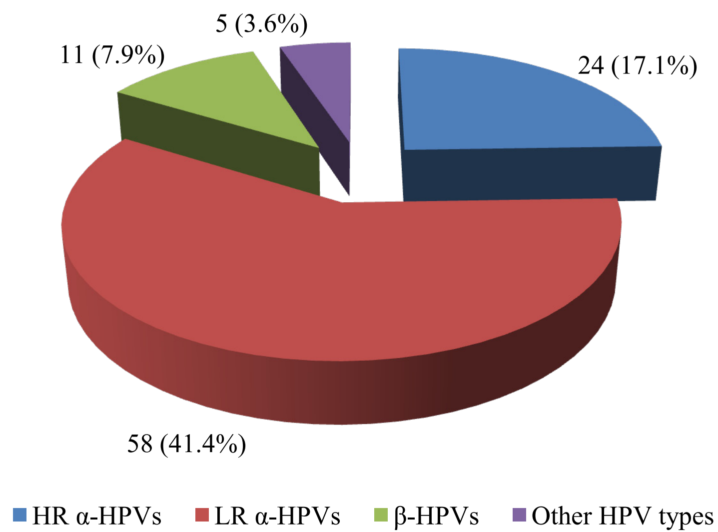



**Figure 6.2: The distribution of HPV types detected**

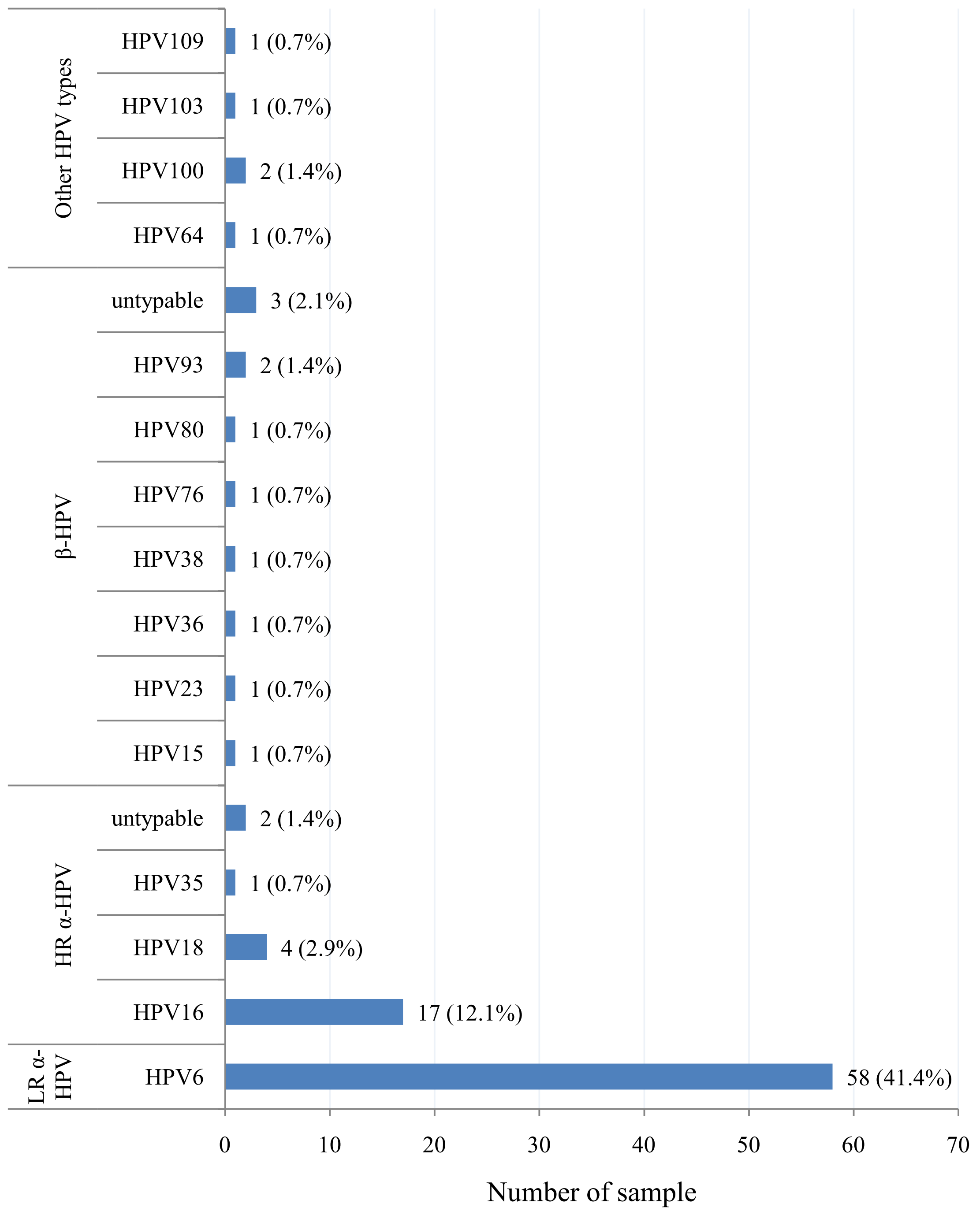



**Figure 6.3: The distribution of HPV types detected**

Table 6.3 illustrates the distribution of HPV types across demographic and clinical characteristics. Appendix 53 (Table A) shows results of Pearson's Chi-Square test for HPV prevalence related to dichotomous variables in demographic and clinicopathological parameters including gender, age group, disease status and disease outcome. Appendix 53 (Table C) demonstrates results of Cramer's V test for HPV prevalence related to larger crosstabs in demographic and clinicopathological parameters which consist of anatomic site, tumour size and regional lymph nodes.

It was found that significantly more females were positive for HR-HPV than other types. Nevertheless the distribution of LR-HPV types was nearly comparable in both sexes. HPV16 type was predominantly present within the HR-HPV group in females, ($\chi^2$=6.14, *df*=1, *N*=140, $p<0.05$). LR-HPV types was statistically more frequently detected in older individuals compared to their counterparts, ($\chi^2$=4.36, *df*=1, *N*=140, $p<0.05$). The vast majority of HPVs (pooled data of LR-HPV and HR-HPV) were identified among patients with no disease recurrence 73/82 (89.0%), appeared to reflect its proportion in the study 129/140 (92.1%). Within HR-HPV, HPV16 was detected relatively more in sample from living patients ($\chi^2$=7.32, *df*=1, *N*=140, $p<0.01$).

**Table 6.3: The distribution of HR-HPV and LR-HPV types within demographic characteristics**

| | HR α-HPV (%) | | | | LR α-HPV (%) |
|---|---|---|---|---|---|
| | HPV16 | HPV18 | HPV35 | Untypable | HPV6 |
| **Gender:** | | | | | |
| Female | 14 (82.4) | 1 (25) | - | 1 (50) | 30 (51.7) |
| Male | 3 (17.6) | 3 (75) | 1 (100) | 1 (50) | 28 (48.3) |
| **Age group:** | | | | | |
| 36 – 64y | 12 (70.6) | 3 (75) | 1 (100) | - | 25 (43.1) |
| 65 – 99y | 5 (29.4) | 1 (25) | - | 2 (100) | 33 (56.9) |
| **Disease status:** | | | | | |
| No recurrence | 15 (88.2) | 4 (100) | 1 (100) | 2 (100) | 51 (87.9) |
| Recurrence | 2 (11.8) | - | - | - | 7 (12.1) |
| **Disease outcome:** | | | | | |
| Alive | 15 (11.8) | 2 (50) | 1 (100) | 1 (50) | 31 (53.4) |
| Dead | 2 (88.2) | 2 (50) | - | 1 (50) | 27 (46.6) |
| **Anatomic sites:** | | | | | |
| Buccal mucosa | - | - | - | - | 3 (5.2) |
| Floor of the mouth | 1 (5.9) | 1 (25) | - | 1 (50) | 10 (17.2) |
| Alveolus | 1 (5.9) | - | - | - | 3 (5.2) |
| Gingivae | - | 1 (25) | - | - | 2 (3.4) |
| Soft palate | 10 (58.8) | - | - | - | 8 (13.8) |
| Pharynx | - | - | - | - | 1 (1.7) |
| Lower lip | - | - | - | - | 3 (5.2) |
| Retromolar pad | 2 (11.8) | - | - | - | 7 (12.1) |
| Supraglottis | - | - | - | - | 1 (1.7) |
| Tongue | 3 (17.6) | 2 (50) | 1 (100) | 1 (50) | 20 (34.5) |
| Tonsil | - | - | - | - | - |
| **Tumour size:** | | | | | |
| Negative | - | - | - | - | 1 (1.7) |
| $T_1$ | 14 (82.4) | 2 (50) | - | 1 (50) | 22 (37.9) |
| $T_2$ | 2 (11.8) | 1 (25) | 1 (100) | - | 14 (24.1) |
| $T_3$ | - | - | - | - | 7 (12.1) |
| $T_4$ | 1 (5.6) | 1 (25) | - | 1 (50) | 14 (24.1) |
| **Regional lymph nodes:** | | | | | |
| Negative | 10 (58.8) | 2 (50) | - | - | 19 (32.8) |
| $N_0$ | 5 (29.4) | 1 (25) | 1 (100) | - | 18 (31.0) |
| $N_1$ | - | 1 (25) | - | 2 (100) | 8 (13.8) |
| $N_2$ | 2 (11.8) | - | - | - | 13 (22.4) |
| **Subtotal** | **17 (70.8)** | **4 (16.7)** | **1 (4.2)** | **2 (8.3)** | **58 (100.0)** |
| **TOTAL (*N* = 82)** | | **24 (29.3)** | | | **58 (70.7)** |

The distribution of LR-HPV identified was scattered across all anatomic sites concerned in this cohort except for tonsil. Within HR-HPV types, HPV16 was the only type detected in alveolus, soft palate and retromolar pad. None of the HR-HPV types were identified in buccal mucosa, pharynx, lower lip, supraglottis and tonsil. Even though, the available samples were dominated by floor of the mouth, tongue and soft palate for this cohort, but the number of different HPV types detected was led by the tongue. Results from Cramer's V test revealed that the prevalence of all HPV types was not statistically significantly different across the anatomic sites.

Similar Cramer's V results were observed for tumour size as for anatomic site but that was not the case for HPV6 ($\chi^2$=0.30, $df$=4, $N$=140, $p$<0.05) and HPV16 ($\chi^2$=0.27, $df$=4, $N$=140, $p$<0.05). Generally, there was a very heterogeneous distribution of all HPV types observed and predominantly with HPV6 in most tumour sizes especially $T_4$, 14/18 (77.8%) and $T_3$, 7/14 (50%). Although the initial samples at $T_2$ were more than double compared to $T_4$, the final frequency of HPV6 detected was comparable between these two tumour sizes. HPV16 and HPV18 were identified of tumour size $T_1$, $T_2$ and $T_4$ but the former was predominant at $T_1$.

A very typical overall HPV prevalence was observed where the percentage of HPV types detected was directly proportional to the level of regional lymph nodes, 37/71 (52%) for negative, 28/35 (80%) for $N_0$, 15/16 (94%) for $N_1$ and 18/18 (100%) for $N_2$. The present of HPV6 was distinctly distributed mainly at $N_0$ and $N_2$ of regional lymph nodes compared to other HPV types ($\chi^2$=0.33, $df$=4, $N$=140, $p$<0.01). Within HR-HPV types detected, the frequency of HPV16 appeared to nearly reflect the initial

proportion of samples according to the level of regional lymph nodes. None of the other HPV types were detected in the positive lymph nodes cases.

### 6.4 p16 EXPRESSION IN ORAL MUCOSA

The same cohort of 140 specimens (as mentioned in section 6.1) was semi-quantitatively assessed for p16 by immunohistochemistry until consensus agreements were achieved among observers. A total of 107 cases (76.4%) were positive for p16 (Figure 6.4). The results of overall p16 staining intensity in this cohort are shown in Figure 6.5. The proportion of positively scored for p16 staining are as follows, 41/140 (29.3%) for 1+, 55/140 (39.3%) for 2+ and 11/140 (7.8%) for 3+.

Appendix 53 (Table B) shows results of Pearson's Chi-Square test for p16 positivity related to dichotomous variables in demographic and clinicopathological parameters including gender, age group, disease status and disease outcome. Since p16 staining intensity had four levels, Cramer's V test was more appropriate for this parameter. Appendix 53 (Table D) demonstrates results of Cramer's V test for both p16 status related to larger crosstabs in clinicopathological parameters which consist of anatomic site, tumour size and regional lymph nodes.

p16 positivity was significantly higher in males ($\chi^2$=8.02, $df$=1, $N$=140, $p$<0.01), older patients ($\chi^2$=8.55, $df$=1, $N$=140, $p$<0.01) and samples taken from deceased patients ($\chi^2$=15.96, $df$=1, $N$=140, $p$<0.001) compared with their counterparts. The p16 positivity was significantly different across anatomic sites (*Cramer's* $V$=0.41, $df$=10, $N$=140, $p$<0.01) and tumour size (*Cramer's* $V$=0.37, $df$=4, $N$=140, $p$<0.01). Further analyses

using Kruskal-Wallis H and Mann-Whitney U were carried out to compare p16 positivity across anatomic site and tumour size. Since only single samples were available for each subsite, we excluded pharynx, supraglottis and tonsil for gross subsite comparison. We found that p16 positivity was significantly lower in soft palate compared each with floor of the mouth ($p$=0.020), lower lip ($p$=0.042) and the tongue ($p$=0.00). The alveolus was significantly lower in p16 positivity compared with the tongue ($p$=0.025). The rest of the subsite comparisons were not significantly different. p16 positivity was significantly lower in $T_1$ compared with $T_2$ ($p$=0.001) and $T_3$ ($p$=0.004).

p16 staining intensity was significantly different between genders (*Cramer's V*=0.26, *df*=3, *N*=140, $p<0.05$), age group (*Cramer's V*=0.30, *df*=3, *N*=140, $p<0.01$), disease outcome (*Cramer's V*=0.40, *df*=3, *N*=140, $p<0.001$) and within tumour size (*Cramer's V*=0.31, *df*=4, *N*=140, $p<0.001$). Further analyses using Mann-Whitney U were carried out to compare p16 staining intensity between gender, age group and disease outcome. Kruskal-Wallis and followed by Mann-Whitney U tests were used for comparison within tumour size. We found that p16 staining intensity was significantly higher in males ($p$=0.004), older patients ($p$=0.001) and deceased patients ($p$=0.000). p16 staining intensity was significantly lower in $T_1$ compared each with $T_2$ (p=0.000) and $T_3$ ($p$=0.000); $T_2$ was significantly lower compared with $T_3$ ($p$=0.047). At this point, further correlation analysis would confirm an association between p16 staining intensity with increasing tumour size. No significant difference for p16 staining intensity was observed in disease status, anatomic site and regional lymph nodes.

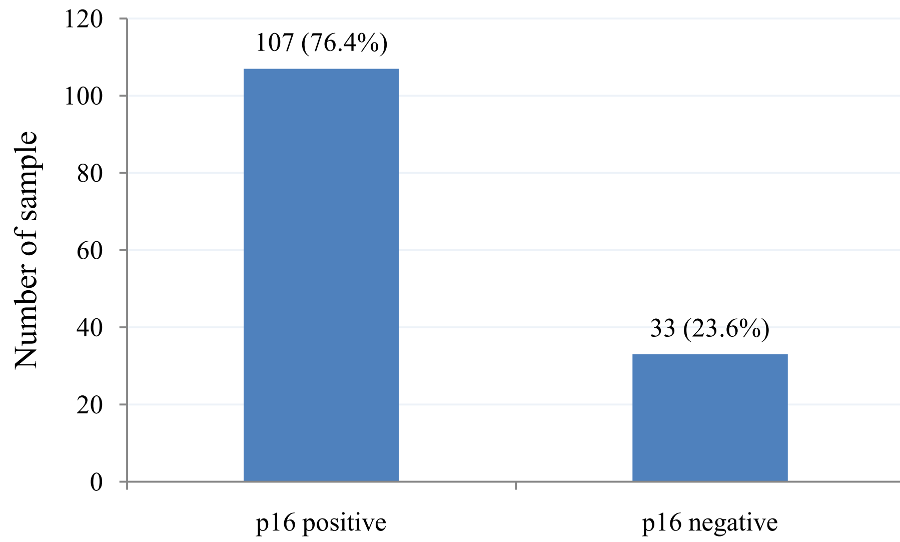



**Figure 6.4: Overall p16 positivity in this cohort**

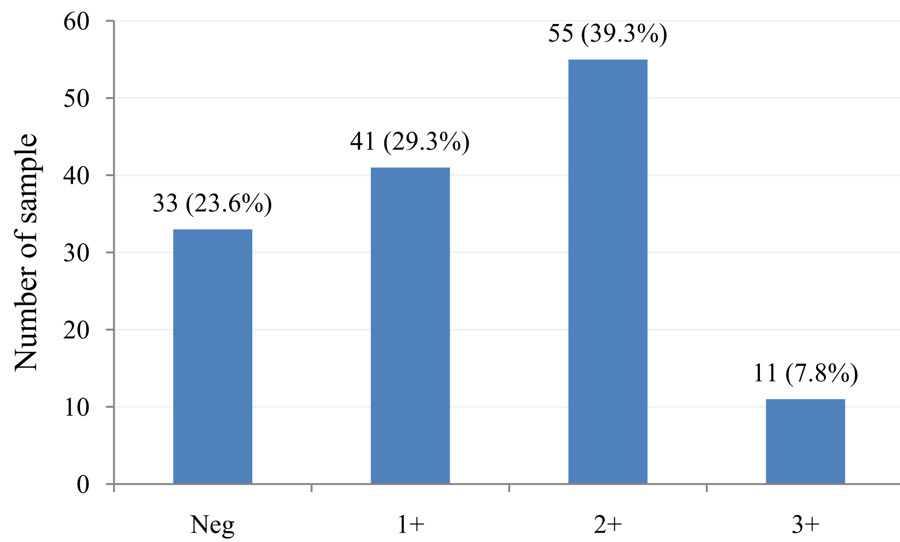



**Figure 6.5: Overall p16 staining intensity in this cohort**

## 6.5 CORRELATION ANALYSES

Correlation analysis was carried out in searching for any association that may exist as follows:

- Relationship within demographic and clinicopathological parameters, HPVs and p16 status
- Relationship between demographic and clinicopathological parameters and HPVs status
- Relationship between demographic and clinicopathological parameters and p16 status
- Relationship between HPVs and p16 status

Table 6.4 shows a significant association within demographic and clinicopathological parameters. It was found that disease outcome negatively correlated with gender, age group, anatomic site and tumour size. On the contrary, anatomic site was positively associated with gender, tumour size and regional lymph nodes. Similarly, tumour size was positively linked with gender and regional lymph nodes. None of the associations could relate disease recurrence with other demographic and clinicopathological parameters. Overall associations above could be summarised using schematic representation as illustrated in Figure 6.6. The positive correlation within HPVs status was transformed into a diagrammatic representation as shown in Figure 6.7. The correlation between p16 status appeared to be straight forward by overlapping of two circles to indicate significant correlation between p16 positivity and p16 staining intensity. Table 6.4 shows significant association between demographic and clinicopathological parameters and HPVs and p16. Most of the parameters compared were positively associated except for disease outcome in which gender, age group, anatomic site and tumour size were negatively correlated.

**Table 6.4: Significant correlations within demographic and clinical parameters, HPVs and p16 status**

| Parameter 1 | Parameter 2 | Pearson Correlation | | Spearman's rho | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Correlation coefficient | Sig. (2-tailed) |
| **Within demographic/clinicopathological parameters:** | | | | | |
| Disease outcome | Gender | -0.262** | 0.002 | -0.262** | 0.002 |
| | Age group | -0.308** | 0.000 | -0.308** | 0.000 |
| | Anatomic site | -0.252** | 0.003 | -0.253** | 0.003 |
| | Tumour size | -0.383** | 0.000 | -0.472** | 0.000 |
| Anatomic site | Gender | 0.179* | 0.035 | 0.173* | 0.040 |
| | Tumour size | 0.354** | 0.000 | 0.071 | 0.407 |
| | Regional Lymph Nodes | 0.287** | 0.001 | 0.238** | 0.005 |
| Tumour size | Gender | 0.172* | 0.042 | 0.220** | 0.009 |
| | Regional Lymph Nodes | 0.354** | 0.000 | 0.344** | 0.000 |
| Recurrence | No association | - | - | - | - |
| **Within HPVs status:** | | | | | |
| Overall HPV status | α-HPV status | 0.853** | 0.000 | 0.853** | 0.000 |
| | HR-HPV | 0.383** | 0.000 | 0.383** | 0.000 |
| | LR-HPV | 0.707** | 0.000 | 0.707** | 0.000 |
| | HPV16 | 0.313** | 0.000 | 0.313** | 0.000 |
| α-HPV status | HR-HPV | 0.448** | 0.000 | 0.448** | 0.000 |
| | LR-HPV | 0.771** | 0.000 | 0.771** | 0.000 |
| | HPV16 | 0.366** | 0.000 | 0.366** | 0.000 |
| HR-HPV | HPV16 | 0.817** | 0.000 | 0.817** | 0.000 |
| **Within p16 status:** | | | | | |
| p16 positivity | p16 staining intensity | 0.794** | 0.000 | 0.775** | 0.000 |

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

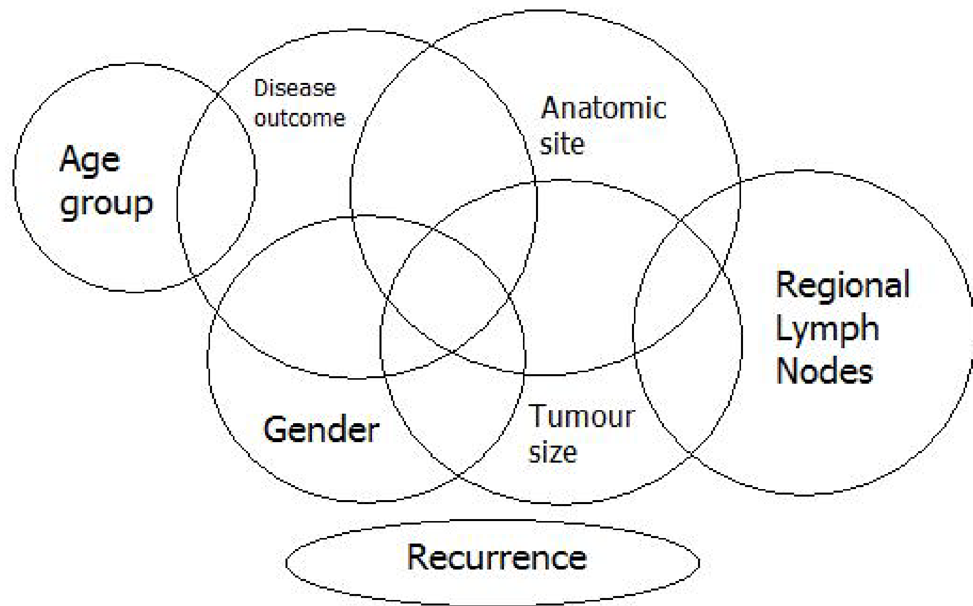



**Figure 6.6: Schematic representation of correlations within demographic and clinicopathological parameters**.

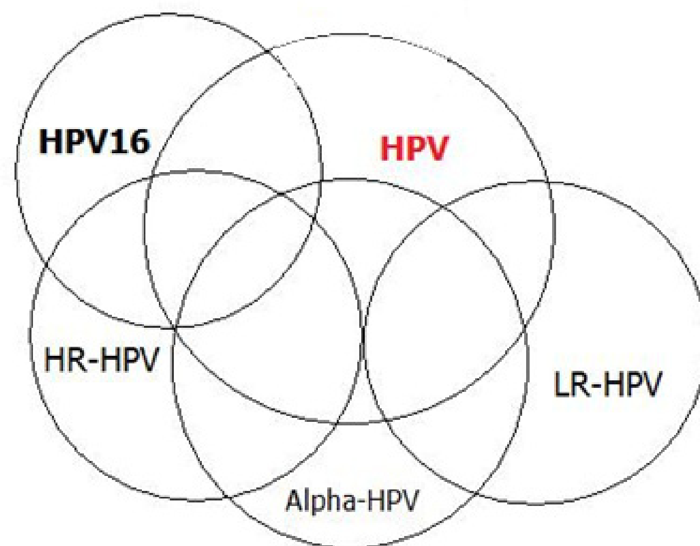



**Figure 6.7: Schematic representation of correlations within HPV status**.

Table 6.5 shows a significant correlation related to each HPVs and p16 status with demographic and clinicopathological parameters. For the former, its association could be categorised into two major groups. The first group was positively associated in all cases and the second group, the association was positive in one case but negative in the other case. HPV, α-HPV and LR-HPV were in the first group whereas HR-HPV and HPV16 were in the second group. The actual association was more complex but was simplified for the purpose of visualisation. Figure 6.8 shows a schematic representation of all the associations between demographic/clinicopathological parameters related to HPVs status. Figure 6.9 illustrates all the associations between demographic/clinico-pathological parameters related to p16 status.

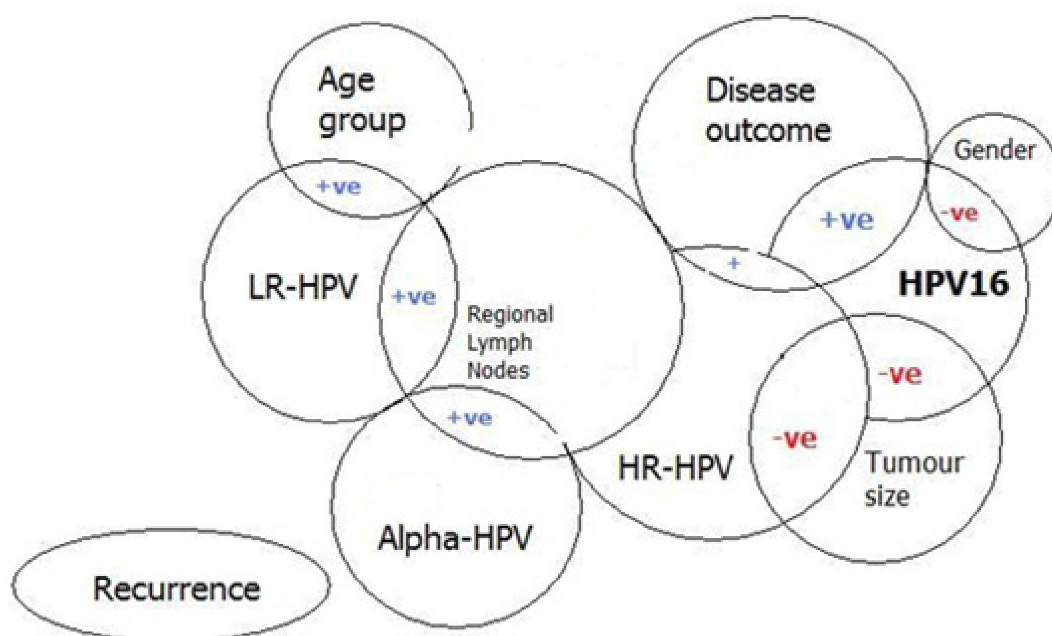



**Figure 6.8: Schematic representation of correlations within demographic and clinicopathological parameters**. The sign (+ve) to represent positive association and (-ve) for negative association.

**Table 6.5: Significant correlations between demographic and clinical parameters; HPVs and p16 status**

| Parameter 1 | Parameter 2 | Pearson Correlation | | Spearman's rho | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Correlation coefficient | Sig. (2-tailed) |
| **Between demographic/clinical parameters and HPVs status:** | | | | | |
| Tumour size | HPVs | 0.166* | 0.049 | 0.129 | 0.130 |
| | HR-HPV | -0.139 | 0.102 | -0.176* | 0.037 |
| | LR-HPV | 0.259** | 0.002 | 0.217* | 0.010 |
| | HPV16 | -0.201* | 0.018 | -0.232** | 0.006 |
| Regional Lymph Nodes | HPVs | 0.180* | 0.033 | 0.179* | 0.035 |
| | α-HPV | 0.157 | 0.063 | 0.168* | 0.048 |
| | LR-HPV | 0.314** | 0.000 | 0.320** | 0.000 |
| Disease outcome | HR-HPV | 0.196* | 0.020 | 0.196* | 0.020 |
| | HPV16 | 0.229** | 0.007 | 0.229** | 0.007 |
| Age group | LR-HPV | 0.177* | 0.037 | 0.177* | 0.037 |
| Gender | HPV16 | -0.209* | 0.013 | -0.209* | 0.013 |
| Recurrence | No association | - | - | - | - |
| **Between demographic/clinical parameters and p16 status:** | | | | | |
| Tumour size | p16 positivity | 0.247** | 0.003 | 0.299** | 0.000 |
| | p16 staining intensity | 0.279** | 0.001 | 0.356** | 0.000 |
| Anatomic site | p16 positivity | 0.179* | 0.034 | 0.170* | 0.044 |
| | p16 staining intensity | 0.203* | 0.016 | 0.184* | 0.030 |
| Disease outcome | p16 positivity | -0.338** | 0.000 | -0.338** | 0.000 |
| | p16 staining intensity | -0.401** | 0.000 | -0.401** | 0.000 |
| Age group | p16 positivity | 0.247** | 0.003 | 0.247** | 0.003 |
| | p16 staining intensity | 0.290** | 0.001 | 0.292** | 0.000 |
| Gender | p16 positivity | 0.239** | 0.004 | 0.239** | 0.004 |
| | p16 staining intensity | 0.248** | 0.003 | 0.245** | 0.004 |
| Recurrence | No association | - | - | - | - |

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

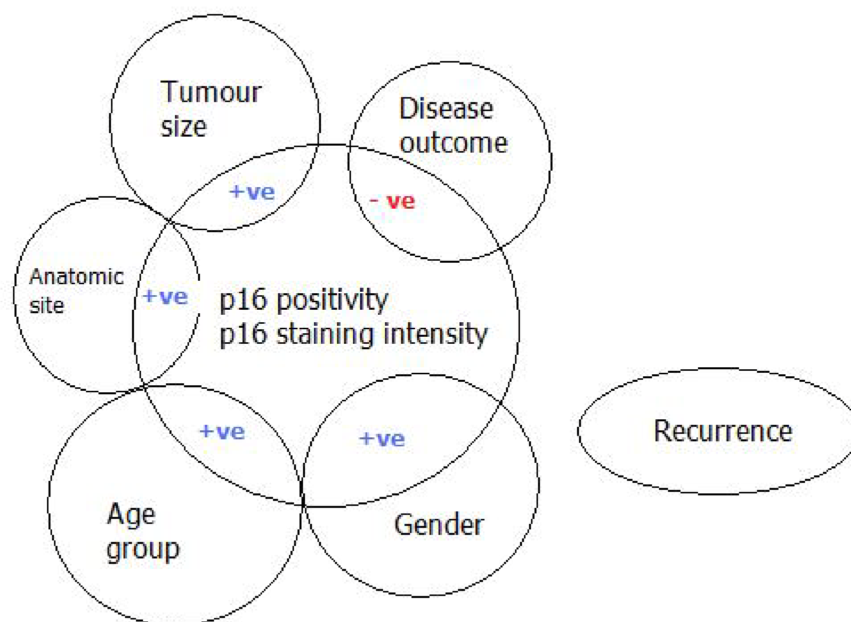



**Figure 6.9: Schematic representation of correlations between demographic/clinicopathological parameters and p16 status**. The sign (+ve) to represent positive association and (-ve) for negative association.

**Table 6.6: Correlation analyses between HPVs and p16 status**

| Parameter 1 | Parameter 2 | Pearson Correlation | | Spearman's rho | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Correlation coefficient | Sig. (2-tailed) |
| **Between HPVs and p16 status:** | | | | | |
| Overall HPVs status | p16 positivity | -0.125 | 0.140 | -0.125 | 0.140 |
| | p16 staining intensity | -0.060 | 0.485 | -0.056 | 0.509 |
| α-HPV status | p16 positivity | -0.110 | 0.196 | -0.110 | 0.196 |
| | p16 staining intensity | -0.098 | 0.248 | -0.091 | 0.287 |
| HR-HPV status | p16 positivity | -0.149 | 0.078 | -0.149 | 0.078 |
| | p16 staining intensity | -0.094 | 0.271 | -0.098 | 0.250 |
| LR-HPV status | p16 positivity | 0.023 | 0.788 | 0.023 | 0.788 |
| | p16 staining intensity | 0.012 | 0.886 | 0.020 | 0.814 |
| HPV16 status | p16 positivity | **-0.206*** | 0.015 | **-0.206*** | 0.015 |
| | p16 staining intensity | -0.127 | 0.134 | -0.135 | 0.111 |

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

Table 6.6 shows the results of correlation analyses between HPVs and p16 status. None of the parameters compared demonstrated any significant correlation except for HPV16 status and p16 positivity. Both parameters were negatively associated (*r*=-0.206, *N*=140, *p*<0.05). Further analyses were carried out searching for an in depth association between p16 and HPVs status and only three parameters, gender, age group and disease status did produce significant correlation as shown in Table 6.8.

**Table 6.7: Correlation analyses between p16 positivity and HPV status**

| Parameter 1 | Parameter 2 | Male (N=64) | | Female (N=76) | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Pearson Correlation | Sig. (2-tailed) |
| p16 positivity | Overall HPV status | 0.060 | 0.639 | **-0.239*** | 0.037 |
| | α-HPV status | 0.000 | 1.000 | -0.178 | 0.125 |
| | LR-HPV status | -0.048 | 0.709 | 0.050 | 0.669 |
| | HPV16 status | 0.084 | 0.510 | **-0.245*** | 0.033 |

| Parameter 1 | Parameter 2 | Age 36-64y (N=75) | | Age 65-99y (N=65) | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Pearson Correlation | Sig. (2-tailed) |
| p16 positivity | Overall HPV status | **-0.228*** | 0.049 | -0.007 | 0.953 |
| | α-HPV status | **-0.245*** | 0.034 | -0.099 | 0.431 |
| | HR-HPV status | **-0.253*** | 0.028 | 0.140 | 0.265 |
| | LR-HPV status | -0.040 | 0.733 | 0.006 | 0.964 |
| | HPV16 status | **-0.309**** | 0.007 | 0.108 | 0.391 |

| Parameter 1 | Parameter 2 | No recurrence (N=129) | | Recurrence (N=11) | |
|---|---|---|---|---|---|
| | | Pearson Correlation | Sig. (2-tailed) | Pearson Correlation | Sig. (2-tailed) |
| p16 positivity | Overall HPV status | -0.160 | 0.070 | 0.418 | 0.200 |
| | α-HPV status | -0.148 | 0.094 | 0.418 | 0.200 |
| | HR-HPV status | -0.169 | 0.055 | 0.149 | 0.662 |
| | LR-HPV status | -0.013 | 0.885 | 0.418 | 0.200 |
| | HPV16 status | **-0.240**** | 0.006 | 0.149 | 0.662 |

*. Correlation is significant at the 0.05 level (2-tailed);
**. Correlation is significant at the 0.01 level (2-tailed).

Only Pearson's correlation results are shown in Table 6.7 as Spearman's rho produced exactly the same value. No significant association related to p16 staining intensity and HPV status was observed. In depth correlation results are about to confirm that negative association between p16 positivity and HPV16 status significantly related to females ($r$=-0.245, $p$<0.05), young patients ($r$=-0.309, $p$<0.01) and patients with no disease recurrence ($r$=-0.240, $p$<0.01). In addition, p16 positivity was negatively correlated with overall HPV status in females ($r$=-0.239, $p$<0.05) and young patients ($r$=-0.228, $p$<0.05). In young patients, α-HPV and HR-HPV were negatively associated with p16 positivity, ($r$=-0.245, $p$<0.05) and ($r$=-0.253, $p$<0.05), respectively.

**Table 6.8: Cross-tabulation of HPV versus p16 expression**

| HPV expression | p16 expression | | Concordance[α] (%) | Discordance[β] (%) | κ |
|---|---|---|---|---|---|
| | p16 negative (%) | p16 positive (%) | | | |
| All samples ($N$ = 140) | | | | | |
| Overall HPV negative | 10 (30.3) | 48 (44.9) | 49.3 | 50.7 | -0.12 |
| Overall HPV positive | 23 (69.7) | 59 (55.1) | | | |
| α-HPV negative | 13 (39.4) | 56 (52.3) | 45.7 | 54.3 | -0.09 |
| α-HPV positive | 20 (60.6) | 51 (47.7) | | | |
| HR α-HPV negative | 24 (72.7) | 92 (86.0) | 27.9 | 72.1 | -0.07 |
| HR α-HPV positive | 9 (27.3) | 15 (14.0) | | | |
| LR α-HPV negative | 20 (60.6) | 62 (57.9) | 46.4 | 53.6 | 0.018 |
| LR α-HPV positive | 13 (39.4) | 45 (42.1) | | | |
| HPV16 negative | 25 (75.8) | 98 (91.6) | **24.3** | **75.7** | -0.081 |
| HPV16 positive | 8 (24.2) | 9 (8.4) | | | |

[α](Concordance positive and concordance negative)x100/total specimens, $N$; [β] – 100% - Concordance

Table 6.8 illustrates the results of Cohen's Kappa test for reliability or agreement between HPV and p16 expression. We demonstrated a very weak agreement relating LR α-HPV status to p16 expression since the kappa value must be positive for reliability measures. The percentage of concordance ranges from 29% to 46%. The highest percentage of discordance was 75.7% relating HPV16 to p16 expression. This results seemed consistent with the previous correlation analyses which demonstrated that HPV16 was negatively associated with p16 expression ($r$=-0.206, $N$=140, $p$<0.05) as previously shown in Table 6.6.

## 6.6 CONCLUSIONS

Our results thus far from the oral disease progression study could conclude:

1. **HPV prevalence comparison by demographic and clinicopathological parameters.**
    - The overall HPV prevalence was 82/140 (58.6%).
    - We have postulated the presence of single, double and multiple HPV infections within a single specimen.
    - 41% of LR-HPV, 17% of HR-HPV and 11% of other HPV types were detected regardless the mode of HPV infections.
    - HPV16 was predominantly present in female patients.
    - LR-HPV type, HPV6 was commonly detected in older patients.
    - HR-HPV types or HPV16 were frequently identified in patients who still survived.
    - Overall HPV prevalence (except for HPV6 and HPV16) was not significant different across anatomic site and tumour size.

- HPV6 was predominantly present across the regional lymph nodes compared to the rest of HPV types concerned.

**2. p16 status comparison by demographic and clinicopathological parameters.**

- It was reported that 107/140 cases (76.4%) were positive for p16.
- The overall score of p16 staining intensity in this cohort are as follows: 33% negative, 29% for 1+, 39% for 2+ and 8% for 3+.
- p16 positivity and p16 staining intensity were significant higher in samples taken from males, older patients and deceased patients compared with their counterparts.
- p16 positivity was significant different across anatomic site and tumour size.
- Individual comparison of selected subsites demonstrated that p16 positivity was significantly lower in soft palate compared to floor of the mouth, lower lip and the tongue; p16 positivity in alveolus was significantly lower than the tongue.
- According to tumour size, p16 positivity was significantly higher in $T_2$ and $T_3$ compared each with $T_1$.
- p16 staining intensity was significantly higher in males, older patients and deceased patients.
- p16 staining intensity was significantly higher in $T_3$ compared with $T_2$; $T_3$ and $T_2$ were significantly higher compared each with $T_1$.
- No significant difference was observed in p16 staining intensity for disease status, anatomic site and regional lymph nodes.

### 3. An association between HPV and p16 with carcinomas

- Within demographic and clinicopathological parameters, a positive association was observed as follows: anatomic site with gender, tumour size and regional lymph nodes; tumour size with gender and regional lymph nodes.
- In contrast, a negative association was observed between disease outcome and gender, age group, anatomic site and tumour size.
- No correlation was found between disease recurrence and other parameters.
- Generally, a positive correlation was observed between different HPV types by their status (the presence/the absence of HPV types in the specimens).
- Similarly, a positive association was found within p16 status.
- A positive association was observed related to regional lymph nodes with overall HPV, α-HPV and LR-HPV status; age group with LR-HPV; HR-HPV/HPV16 with disease outcome.
- A negative association was observed related to tumour size with HR-HPV/HPV16 status.
- A positive association was observed related to p16 positivity/p16 staining intensity with tumour size, anatomic site, age group and gender.
- A negative association was observed relating p16 positivity/p16 staining intensity with disease outcome.
- Finally, we found that **HPV16 expression was negatively associated with p16 expression**.
- In depth correlation results supported a negative association between p16 positivity and HPV16 status, significantly related to female, young patients and patients with no disease recurrence.

# CHAPTER 7

# THE PROGNOSTIC IMPLICATION OF HPV AND p16

## 7.1 OVERVIEW

An overview of the aims of this chapter is as follows:

- To determine potential risk factors associated with patients overall survival (OS) and recurrence free-survival (RFS) using Kaplan-Meier survival estimates.
- To evaluate the significant prognosticators and other related confounders by univariate and multivariate analysis on OS and RFS using Cox regression model.
- To perform individual analyses of HPV and p16 status and followed by combined HPV/p16 status using Cox proportional hazard models with adjustments to assess their prognostic value.
- To assess the prognostic implication of HPV and p16 in head and neck cancer.

## 7.2 SURVIVAL ANALYSIS AND COX PROPORTIONAL HAZARD MODELS

Survival was measured in months from the date of diagnosis until death or until the patient was last known to be alive. Survival rates for OS and RFS were estimated according to the Kaplan-Meier survival estimates. Continuous data values were divided by the median and all survival curves were generated in SPSS ver.16. A $p$ value $\leq 0.05$

was considered significant. The Kaplan-Meier curve of the survival followed by Log Rank (Mantel-Cox) test was used to investigate whether there was an association between the clinical parameters and patients survival. Since the above technique is the univariate version of survival analysis, later by performing Cox regression analysis it will further assist the actual effect of confounders on the survival of the patient. For OS and RFS, univariate and multivariate analysis were performed by Cox regression using the "enter" method. All clinicopathologic variables significant in univariate analysis to OS and RFS were adjusted for multivariate analysis. The Wald statistic and hazard ratio with 95% confidence interval (CI) were used to assess risk of molecular alterations and reference categories were selected as the lowest risk categories for prognostic variables.

These analyses included gender, age group, anatomic site, tumour size and regional lymph nodes, survival and disease recurrence also were examined in separate models. Test for interactions among risk factors were generated using both logistic regression and proportional hazard regression methods. All variables were assessed for the proportional hazard assumption before inclusion in the models. Cox proportional hazard models were used to assess the relationships between HPV and p16 and to adjust for factors previously found to be prognostically significant in cancer. For HPV status which consisted of overall HPV types (we will refer as HPV for short), α-HPV types, high risk HPV (HR-HPV), low risk HPV (LR-HPV) and HPV16. Meanwhile, p16 positivity and p16 staining intensity were the selected parameters to represent p16 status. An assessment was carried out separately and then four groups for instance combined HPV/p16 status: HPV/p16+ (reference group), HPV/p16-negative, HPV-negative/p16+, HPV-negative/p16-negative. The magnitude of the associations was assessed with hazard ratios (HRs) calculated from the Cox regression models. The

significant level was based on two-tailed tests and *p*-values ≤ 0.05. Statistical analyses were performed using SPSS 16.0.

A total of 140 patients were included in the survival analysis. For OS, the median was 38 months, with range of 7.60 to 68.40 months; for RFS, it was 60 months, with a range of 59.45 to 60.55 months. Univariate and multivariate survival analysis was performed to identify factors with an independent effect on risk of death and disease recurrence from head and neck cancer using Cox proportional hazard regression model. Table 7.1 and Table 7.2 show assessment results from Kaplan-Meier test for OS and RFS, respectively.

**Table 7.1: The prognostic significance risk factors on OS from Kaplan-Meier test**

| Parameter | Log Rank (Mantel-Cox) | | |
|---|---|---|---|
| | *Chi-Square* | *df* | *Sig.* |
| Gender | 8.575** | 1 | 0.003 |
| Age group | 12.155** | 1 | 0.000 |
| Anatomic site | 31.124** | 8 | 0.000 |
| Tumour size | 49.058** | 2 | 0.000 |
| Regional Lymph Nodes | 6.878* | 2 | 0.032 |
| Overall HPVstatus | 0.546 | 1 | 0.460 |
| α-HPV status | 0.111 | 1 | 0.739 |
| High risk HPV status | 4.244* | 1 | 0.039 |
| Low risk HPVstatus | 1.287 | 1 | 0.257 |
| HPV16 status | 5.884* | 1 | 0.015 |
| p16 status | 14.718** | 1 | 0.000 |
| p16 staining intensity | 18.838** | 1 | 0.000 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

**Table 7.2: The prognostic significance risk factors on RFS from Kaplan-Meier test**

| Parameter | Log Rank (Mantel-Cox) | | |
|---|---|---|---|
| | *Chi-Square* | *df* | *Sig.* |
| Gender | 5.711* | 1 | 0.017 |
| Age group | 3.613 | 1 | 0.057 |
| Anatomic site | 19.493* | 8 | 0.012 |
| Tumour size | 43.306** | 2 | 0.000 |
| Regional Lymph Nodes | 17.396** | 2 | 0.000 |
| Overall HPVstatus | 1.110 | 1 | 0.292 |
| α-HPV status | 0.060 | 1 | 0.806 |
| High risk HPV status | 2.466 | 1 | 0.116 |
| Low risk HPVstatus | 1.456 | 1 | 0.228 |
| HPV16 status | 6.303* | 1 | 0.012 |
| p16 status | 10.535** | 1 | 0.001 |
| p16 staining intensity | 11.214** | 1 | 0.001 |

*N* – number of samples, ** Statistically significant at the 0.01 level and * at the 0.05 level

In total patients, univariate analysis for OS demonstrated that besides gender ($p$=0.005), age group ($p$=0.001), anatomic site ($p$=0.005), tumour size ($p$=0.000) and regional lymph nodes ($p$=0.032), the significant potential prognosticators were high risk HPV ($p$=0.048), HPV16 ($p$=0.029), p16 positivity ($p$=0.001) and p16 staining intensity ($p$=0.000). In Cox multivariate analysis, after adjusting for gender, age group, anatomic site, tumour size and regional lymph nodes, only p16 staining intensity ($p$=0.016) remained a significant predictor of OS (Table 7.3). The Kaplan-Meier survival curve for OS indicated that 39% of patients with higher intensity in p16 staining (2+/3+) group [denoted as p16 (2+/3+)] was significantly associated with patient poor survival compared with 74% of patients with negative/weak (1+) p16 staining [denoted as p16 (neg/1+)] (log-rank = 18.8, $df$ = 1, $p$ = 0.000) (Figure 7.1).

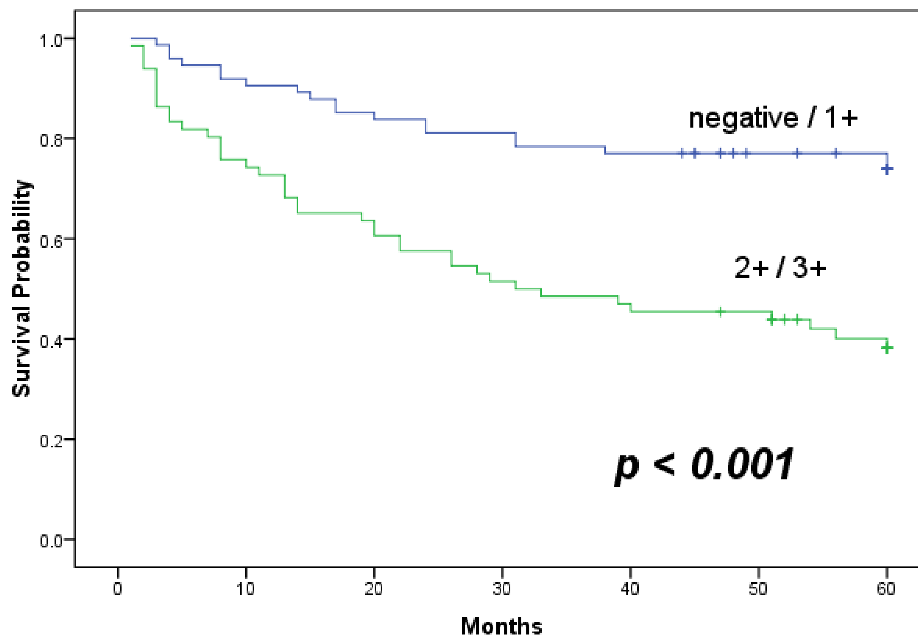



**Figure 7.1: Total patients, significantly worse OS noted in patients with higher intensity of p16 staining (2+/3+) compared with those with p16 negative/weak staining (1+).**

Univariate analysis for RFS in total patients demonstrated that besides tumour size ($p$=0.000) and regional lymph nodes ($p$=0.002), the significant potential prognosticators were p16 positivity ($p$=0.021) and p16 staining intensity ($p$=0.006). In Cox multivariate analysis, after adjusting for tumour size and regional lymph nodes, none of the factors remained significant predictor of RFS (Table 7.4).

**Table 7.3: Univariate and multivariate of OS in total patients (N=140)**

| Variable | HR (95% CI) | *p* value |
|---|---|---|
| **Univariate analysis:** | | |
| Gender | 2.137 (1.265-3.612) | 0.005 |
| Age group | 2.487 (1.458-4.243) | 0.001 |
| Anatomic site | 1.095 (1.028-1.167) | 0.005 |
| Tumour size | 1.649 (1.339-2.031) | 0.000 |
| Regional Lymph Nodes | 1.278 (1.021-1.600) | 0.032 |
| Overall HPVstatus | 1.216 (0.720-2.052) | 0.464 |
| α-HPV status | 1.090 (0.654-1.816) | 0.741 |
| High risk HPV status | 0.397 (0.159-0.994) | 0.048 |
| Low risk HPVstatus | 1.341 (0.803-2.240) | 0.262 |
| HPV16 status | 0.209 (0.051-0.855) | 0.029 |
| p16 positivity | 5.731 (2.074-15.838) | 0.001 |
| p16 staining intensity | 2.015 (1.501-2.705) | 0.000 |
| **Multivariate analysis*:** | | |
| High risk HPV status | 0.884 (0.320-2.446) | 0.813 |
| HPV16 status | 0.856 (0.181-4.043) | 0.844 |
| p16 positivity | 2.197 (0.721-6.694) | 0.166 |
| p16 staining intensity | 1.600 (1.091-2.346) | 0.016 |

*N* – number of patients; HR, hazard ratio; CI, confidence interval.
* Adjusted for gender, age group, tumour size and regional lymph nodes.

**Table 7.4: Univariate and multivariate of RFS in total patients (N=140)**

| Variable | HR (95% CI) | *p* value |
|---|---|---|
| **Univariate analysis:** | | |
| Gender | 0.729 (0.515-1.032) | 0.075 |
| Age group | 1.287 (0.908-1.825) | 0.156 |
| Anatomic site | 1.031 (0.989-1.075) | 0.151 |
| Tumour size | 1.420 (1.217-1.657) | 0.000 |
| Regional Lymph Nodes | 1.287 (1.095-1.514) | 0.002 |
| Overall HPVstatus | 1.149 (0.810-1.630) | 0.437 |
| α-HPV status | 1.033 (0.731-1.458) | 0.856 |
| High risk HPV status | 0.768 (0.485-1.217) | 0.261 |
| Low risk HPVstatus | 1.177 (0.803-2.240) | 0.368 |
| HPV16 status | 0.619 (0.361-1.062) | 0.082 |
| p16 positivity | 1.607 (1.075-2.401) | 0.021 |
| p16 staining intensity | 1.296 (1.077-1.560) | 0.006 |
| **Multivariate analysis*:** | | |
| p16 positivity | 1.337 (0.871-2.052) | 0.185 |
| p16 staining intensity | 1.224 (0.991-1.511) | 0.060 |

*N* – number of patients; HR, hazard ratio; CI, confidence interval.
* Adjusted for tumour size and regional lymph nodes.

There was a drop in the odds ratio associated with p16+ status after inclusion of gender, age group, tumour size, regional lymph nodes, survival and disease recurrence in the adjusted ORs. As a result, males and older patients were no longer statistically significantly associated with p16 expression but living patients remained significant (Table 7.5). Despite a significant drop in the odds ratio associated with p16+ status due to inclusion factors, the risk of tumours of the alveolus remained significantly elevated compared to reference group but it was not the case for lower lip.

**Table 7.5: Demographic and risk factor characteristics of head and neck cancer by p16 status**

| Characteristics | *N* | p16 - *N* (%) | p16+ *N* (%) | Unadjusted OR (95% CI) | Adjusted OR (95% CI) |
|---|---|---|---|---|---|
| Total | 140 | 33 | 107 | | |
| **Gender** | | | | | |
| Female$^{\alpha}$ | 76 | 25 (75,8) | 51 (47.7) | 1.0 | 1.0 |
| Male | 64 | 8 (24.2) | 56 (52.3) | 2.0 (1.3 – 3.3) | 0.8 (0.3 – 2.0) |
| **Age group** | | | | | |
| 36-64y$^{\alpha}$ | 75 | 25 (75.8) | 50 (46.7) | 1.0 | 1.0 |
| 65-99y | 65 | 8 (24.2) | 57 (53.3) | 2.0 (1.2 – 3.2) | 0.5 (0.2 – 1.3) |
| **Anatomic site** | | | | | |
| Pharynx/supraglottis/tonsil$^{\alpha}$ | 3 | - | 3 (2.8) | 1.0 | 1.0 |
| Buccal mucosa | 4 | - | 4 (3.7) | N/E | N/E |
| Floor of the mouth | 22 | 4 (12.1) | 18 (16.8) | N/E | N/E |
| Alveolus | 4 | 2 (6.1) | 2 (1.9) | 4.5 (1.5-13.3) | 1.3 (0.3 – 5.7) |
| Gingivae | 4 | 1 (3.0) | 3 (2.8) | 1.0 | 0.2 (0.01 – 3.3) |
| Soft palate | 37 | 18 (54.5) | 19 (17.8) | 3.0 (0.3-28.8) | 0.1 (0.0 – 1.9) |
| Lower lip | 5 | - | 5 (4.7) | 1.1 (0.6-2.0) | 0.2 (0.0 – 0.8) |
| Retromolar pad | 12 | 3 (9.1) | 9 (8.4) | N/E | N/E |
| Tongue | 49 | 5 (15.2) | 44 (41.1) | 3.0 (0.8-11.1) | 0.6 (0.1 – 4.2) |
| **Tumour size** | | | | | |
| Negative/$T_1$$^{\alpha}$ | 67 | 26 (78.8) | 41 (38.3) | 1.0 | 1.0 |
| $T_2$ | 41 | 4 (12.1) | 57 (34.6) | 1.6 (1.0 – 2.6) | 0.7 (0.2 – 2.1) |
| $T_3$ | 32 | 3 (9.1) | 29 (27.1) | 9.3 (3.3-26.0) | 2.0 (0.5 – 7.9) |
| **Regional Lymph Nodes** | | | | | |
| Negative$^{\alpha}$ | 71 | 20 (60.6) | 51 (47.7) | 1.0 | 1.0 |
| $N_0$ | 35 | 8 (24.2) | 27 (25.2) | 2.6 (1.5 – 4.3) | 1.8 (0.6 – 6.0) |
| $N_1$/$N_2$ | 34 | 5 (15.2) | 29 (27.1) | 3.4 (1.5 – 7.4) | 1.9 (0.6 – 7.0) |
| **Disease outcome** | | | | | |
| Dead$^{\alpha}$ | 59 | 4 (12.1) | 55 (51.4) | 1.0 | 1.0 |
| Alive | 81 | 29 (87.9) | 52 (48.6) | 13.8 (5.0 – 37.9) | 5.5 (1.7 – 17.9) |
| **Disease status** | | | | | |
| No recurrence$^{\alpha}$ | 129 | 32 (97.0) | 97 (90.7) | 1.0 | 1.0 |
| Recurrence | 11 | 1 (3.0) | 10 (9.3) | 3.0 (2.0 – 4.5) | 2.3 (0.7 – 7.1) |

*N* – number of samples, OR - odds ratio $^{\alpha}$ - reference group for odds ratio; N/E – not estimable
Adjusted OR for gender, age group and tumour size, regional lymph nodes, disease outcome and disease status

**Table 7.6: Prognostic significance of risk factors of HPVs by p16 status**

| Characteristics | *N* | p16- *N* (%) | p16+ *N* (%) | Unadjusted OR (95% CI) | Adjusted OR (95% CI) |
|---|---|---|---|---|---|
| Total | 140 | 33 | 107 | | |
| **HPV status** | | | | | |
| Negative[α] | 58 | 10 (30.3) | 48 (44.9) | 1.0 | 1.0 |
| Positive | 82 | 23 (69.7) | 59 (55.1) | 4.8 (2.4 – 9.5) | 2.8 (1.1 – 7.4) |
| **α-HPV status** | | | | | |
| Negative[α] | 69 | 13 (39.4) | 56 (52.3) | 1.0 | 1.0 |
| Positive | 71 | 20 (60.6) | 51 (47.7) | 4.3 (2.4 – 7.9) | 2.3 (0.9 – 5.6) |
| **HR-HPV** | | | | | |
| Negative[α] | 116 | 24 (72.7) | 92 (86.0) | 1.0 | 1.0 |
| Positive | 24 | 9 (27.3) | 15 (14.0) | 3.8 (2.5 – 6.0) | 2.1 (0.8 – 5.4) |
| **LR-HPV** | | | | | |
| Negative[α] | 82 | 20 (60.6) | 62 (57.9) | 1.0 | 1.0 |
| Positive | 58 | 13 (39.4) | 45 (42.1) | 3.1 (1.9 – 5.1) | 1.5 (0.6 – 4.0) |
| **HPV16** | | | | | |
| Negative[α] | 123 | 25 (75.8) | 98 (91.6) | 1.0 | 1.0 |
| Positive | 17 | 8 (24.2) | 9 (8.4) | 3.9 (2.5 – 6.1) | 2.9 (1.0 – 8.4) |

*N* – number of samples, OR odds ratio [α] - reference group for odds ratio;
Adjusted OR for gender, age group and tumour size, regional lymph nodes, disease outcome and disease status

Table 7.6 shows the prognostic significance of the risk factors of head and neck cancer by HPVs and p16 status. HPV and HPV16 status were significantly associated with p16 over-expression after adjustment for gender, age group, tumour size, regional lymph nodes, survival and disease recurrence. The risk remained statistically significantly elevated in the p16+ patients for HPV-positive and HPV16-positive in ascending order of ORs compared to the reference group. Unfortunately, after inclusion of other clinicopathologic characteristics in the adjusted ORs, α-HPV, HR-HPV and LR-HPV were no longer statistically significantly associated with p16 expression.

**Table 7.7: Adjusted hazard ratios for p16/HPV models of survival and recurrence**

| Characteristics | Overall Survival | | Recurrence-Free Survival | |
|---|---|---|---|---|
| | Model 1$^{\beta}$ | Model 2$^{\gamma}$ | Model 1$^{\beta}$ | Model 2$^{\delta}$ |
| **p16 status$^{\beta}$** | | | | |
| p16+ | 0.2 (0.1 – 0.5) | 0.4 (0.1 – 1.0) | 0.6 (0.4 – 0.9) | 0.8 (0.5 – 1.2) |
| p16-$^{\alpha}$ | 1.0 | 1.0 | 1.0 | 1.0 |
| **HPV status$^{\beta}$** | | | | |
| HPV+ | 0.8 (0.5 – 1.4) | 0.8 (0.5 – 1.4) | 0.9 (0.6 – 1.2) | 0.9 (0.6 – 1.3) |
| HPV-$^{\alpha}$ | 1.0 | 1.0 | 1.0 | 1.0 |
| **HPV16 status$^{\beta}$** | | | | |
| HPV16+ | 0.2 (0.1 – 0.9) | 1.5 (0.3 – 6.7) | 0.6 (0.4 – 1.1) | 1.1 (0.6 – 2.0) |
| HPV16-$^{\alpha}$ | 1.0 | 1.0 | 1.0 | 1.0 |
| **HPV/p16 status** | | | | |
| HPV+/p16+$^{\alpha}$ | 1.0 | 1.0 | 1.0 | 1.0 |
| HPV+/p16- | N/E | N/E | 2.1 (1.0 – 4.3) | 1.6 (0.8 – 3.3) |
| HPV-/p16+ | N/E | N/E | 1.3 (0.6 – 2.7) | 1.1 (0.5 – 2.5) |
| HPV-/p16- | N/E | N/E | 1.7 (0.8 – 3.5) | 1.3 (0.6 – 2.8) |
| **HPV16/p16 status** | | | | |
| HPV16+/p16+$^{\alpha}$ | 1.0 | 1.0 | 1.0 | 1.0 |
| HPV16+/p16- | 1.6 (0.3 – 8.7) | 1.8 (0.3 – 10.0) | 0.9 (0.4 – 2.2) | 1.0 (0.4 – 2.4) |
| HPV16-/p16+ | N/E | N/E | 0.9 (0.4 – 1.9) | 1.3 (0.6 – 3.1) |
| HPV16-/p16- | 4.6 (1.6 – 12.6) | 2.6 (0.9 – 7.7) | 1.6 (1.0 – 2.6) | 1.4 (0.9 – 2.4) |

$^{\alpha}$ - reference group for hazard ratio (HR); N/E – not estimable
$^{\beta}$ HRs not adjusted for p16 and HPV, gender, age group, tumour size and regional lymph nodes
$^{\gamma}$ HRs are adjusted for p16 and HPV, gender, age group, tumour size and regional lymph nodes
$^{\delta}$ HRs are adjusted for p16 and HPV, tumour size and regional lymph nodes

**Table 7.7: (Continued)**

| Characteristics | Overall Survival | | Recurrence-Free Survival | |
|---|---|---|---|---|
| | Model 1[β] | Model 2[γ] | Model 1[β] | Model 2[δ] |
| **p16 staining intensity[β]** | | | | |
| Negative/1+[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| 2+/3+ | 3.1 (1.8 – 5.4) | 2.1 (1.2 – 3.8) | 1.6 (1.1 – 2.2) | 1.3 (0.9 – 1.9) |
| **HPV/p16 staining intensity** | | | | |
| HPV+/ p16(Negative/1+)[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| HPV+/p16(2+/3+) | 3.4 (1.6 – 7.3) | 2.3 (1.0 – 5.3) | 1.9 (1.2 – 3.1) | 1.5 (0.9 – 2.6) |
| HPV-/ p16(Negative/1+) | 0.8 (0.3 – 1.9) | 0.9 (0.3 – 2.2) | 1.0 (0.6 – 1.6) | 1.0 (0.6 – 1.6) |
| HPV-/p16(2+/3+) | 2.0 (0.8 – 4.5) | 1.5 (0.6 – 3.6) | 1.3 (0.7 – 2.2) | 1.1 (0.7 – 2.0) |
| **HPV16/p16 staining intensity** | | | | |
| HPV16+/ p16(Negative/1+)[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| HPV16+/p16(2+/3+) | 1.6 (0.4 – 6.8) | 2.1 (0.4 – 9.4) | 0.9 (0.3 – 2.5) | 0.9 (0.3 – 2.5) |
| HPV16-/ p16(Negative/1+) | N/E | N/E | 0.7 (0.4 – 1.3) | 1.0 (0.5 – 2.0) |
| HPV16-/p16(2+/3+) | 2.6 (1.5 – 4.6) | 1.9 (1.0 – 3.5) | 1.5 (1.1 – 2.2) | 1.4 (0.9 -2.2) |
| **Gender** | 0.5 (0.3 – 0.8) | 0.8 (0.4 – 1.3) | 0.7 (0.5 – 1.0) | 1.0 (0.7 – 1.4) |
| **Age group** | 0.4 (0.2 – 0.7) | 0.5 (0.3 – 0.8) | 0.8 (0.5 – 1.1) | 0.9 (0.6 – 1.3) |
| **Tumour size** | | | | |
| Negative/$T_1$[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| $T_2$ | 0.1 (0.1 – 0.3) | 0.2 (0.1 – 0.4) | 0.4 (0.3 – 0.6) | 0.5 (0.2 – 0.7) |
| $T_3$ | 1.3 (0.7 – 2.2) | 1.3 (0.7 – 2.4) | 1.0 (0.6 – 1.6) | 1.0 (0.6 – 1.7) |
| **Regional Lymph Nodes** | | | | |
| Negative[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| $N_0$ | 0.5 (0.3 – 0.8) | 1.0 (0.6 – 1.9) | 0.5 (0.3 – 0.7) | 0.7 (0.4 – 1.1) |
| $N_1/N_2$ | 0.6 (0.3 – 1.2) | 1.2 (0.6 – 2.5) | 0.6 (0.4 – 1.1) | 0.9 (0.5 – 1.5) |
| **Anatomic site** | | | | |
| Pharynx/supraglottis/tonsil[α] | 1.0 | 1.0 | 1.0 | 1.0 |
| Buccal mucosa | 1.7 (0.4 – 7.2) | 1.2 (0.3 – 5.4) | 3.2 (1.0 – 10.3) | 2.1 (0.6 – 7.2) |
| Floor of the mouth | 2.0 (0.6 – 6.6) | 1.5 (0.4 – 5.6) | 1.3 (0.4 – 4.2) | 1.2 (0.3 – 4.2) |
| Alveolus | 0.2 (0.1 – 0.7) | 0.3 (0.1 – 0.8) | 0.7 (0.4 – 1.3) | 0.7 (0.4 – 1.2) |
| Gingivae | 0.4 (0.1 – 2.7) | 0.5 (0.1 – 3.8) | 0.9 (0.3 – 3.0) | 0.9 (0.3 – 3.3) |
| Soft palate | 2.4 (0.8 – 6.8) | 2.2 (0.7 – 6.7) | 1.6 (0.6 – 4.6) | 1.5 (0.5 – 4.4) |
| Lower lip | 0.3 (0.1 – 0.6) | 0.9 (0.4 – 2.2) | 0.6 (0.4 – 1.0) | 1.4 (0.8 – 2.6) |
| Retromolar pad | 1.0 (0.3 – 3.2) | 2.4 (0.6 – 9.4) | 1.1 (0.4 – 2.7) | 2.0 (0.7 – 5.7) |
| Tongue | 0.7 (0.3 – 1.7) | 0.7 (0.3 – 1.8) | 1.0 (0.5 – 2.0) | 1.0 (0.5 – 2.1) |

[α] - reference group for hazard ratio (HR); N/E – not estimable
[β] HRs not adjusted for p16 and HPV, gender, age group, tumour size and regional lymph nodes
[γ] HRs are adjusted for p16 and HPV, gender, age group, tumour size and regional lymph nodes
[δ] HRs are adjusted for p16 and HPV, tumour size and regional lymph nodes

Table 7.7 shows the adjusted hazard ratios (HRs) of OS and RFS for p16 positivity and p16 staining intensity, HPV and HPV16 status. Model 1 represented unadjusted HRs whereas Model 2 for adjusted HRs by appropriate significant confounders determined from the previous analyses. Multivariate analyses demonstrated that gender, age group, tumour size and regional lymph nodes were associated with OS and should be included in the appropriate models. Meanwhile, tumour size and regional lymph nodes were associated with clinical outcomes in RFS were included in the final models. Compared to those with p16 positivity, patients who did not exhibit p16 had significantly better OS and RFS (log rank=14.718, $p$=0.000) and (log rank=10.535, $p$=0.001), respectively. In contrast, patients who did not exhibit HPV16 had significantly worse OS (log rank=5.888, $p$=0.015) and RFS (log rank=6.303, $p$=0.012). There were no significant differences in outcomes for HPV cases in OS and RFS.

Subsequently, we investigated the combined HPV/p16 and HPV16/p16 groups for clinical outcomes in comparison to those based on their previously separated analyses. OS and RFS were significantly worse among those who were HPV+/p16+ (log rank=16.558, $p$=0.001) and (log rank=13.116, $p$=0.004) (Figure 7.2A and 7.2B). Patients who had HPV-/p16- tumours also had better RFS than those who did express p16. HPV-/p16- group would not have been predicted in OS due to the fact they were censored ($n$=10). Even though the HPV+/p16- group was associated with an increased risk of disease recurrence (Model 1, HR = 2.1, 1.0 – 4.3) but adjusted HRs indicated that association was no longer significant (Table 7.7). Patients with HPV-/p16+ had the least elevated risk of disease recurrence compared to the reference group. The risk of death and median time to death was not estimable. The median time to disease recurrence was shorter in HPV+/p16+ (45 months) compared to each of the other

groups: HPV+/p16- (60 months), HPV-/p16+ (54 months) and HPV-/p16- (60 months). HPV+/p16- group had better 5-year OS compared to the other groups: 82.6% versus 52.1% HPV-/p16+ and 45.8%, HPV+p16+. HPV+/p16+ group had better 5-year RFS compared to the other groups: 11.9% versus 10% HPV-/p16-, 0%, HPV+p16- and 6.3%, HPV-/p16+.

Prognosis based on the individual biomarkers, HPV and p16, was compared to those based on the joint group effects. OS and RFS among those who express p16 were worse than among who did not, but for HPV cases were statistically comparable. The distinctions in prognostic outcomes were more apparent once both markers were examined together. Considering p16 status alone failed to differentiate the greater risk of disease recurrence by HPV status among the p16-negative groups (Model 1, HR=1.0 versus HPV/p16, HRs 1.7 and 2.1). No further distinction could be made by examining HPV status alone since its prognostic outcomes were comparable in OS and RFS. The addition of HPV status showed that the risk of disease recurrence was actually greater among those who were HPV+/p16- than among either HPV-/p16- or HPV-/p16+ group.

OS and RFS were significantly worse among those who were HPV16-/p16+ (log rank=18.177, *p*=0.000) and (log rank=15.184, *p*=0.002), respectively (Figure 7.3A and 7.3B). The patterns of OS and RFS were almost similar by HPV16/p16 status except HPV16+/p16- group would not have been predicted in OS as ts they were censored (*n*=8). HPV16-/p16- group had better 5-year OS compared to the other groups: 84.0% versus 77.8% HPV16+/p16+, 45.9%, HPV16-/p16+. HPV16+/p16+ group had better 5-year RFS compared to the other groups: 22.2% versus 8.2% HPV16-/p16+, 4.0%, HPV16-/p16- and 0%, HPV+/p16-. The median time to death was not estimable. The

median time to disease recurrence was shorter in the HPV16-/ p16+ (48 months) compared to each of the other groups: HPV16+/p16+ (60 months), HPV16+/p16- (60 months) and HPV16-/p16- (60 months). Although HPV16-/p16- group had the greatest likelihood of death and disease recurrence but after adjusted HRs, it was no longer significantly associated with OS and RFS. Prognosis based on the individual biomarkers, HPV and p16, was compared to those based on the joint group effects. OS and RFS among those who express HPV16 were better than among who did not. The addition of p16 status showed that the risk of death and disease recurrence was actually lower among HPV16-/p16- group and HPV16+/p16+ group, respectively.

Compared to those with p16 staining intensity, patients who exhibit p16 (negative/1+) had significantly better OS (log rank=18.838, $p$=0.000) and RFS (log rank=11.214, $p$=0.001). Next, we investigated separately p16 staining intensity each with overall HPV and HPV16 groups for clinical outcomes in comparison to those based on their previously separated analyses. OS and RFS were significantly worse among those who were HPV+/p16(Neg/1+) (log rank=23.797, $p$=0.000) and (log rank=15.652, $p$=0.001), respectively (Figure 7.4A and 7.4B). HPV+/p16 (2+/3+) group had better 5-year OS compared to the other groups: 77.3% versus 70.0% HPV-/p16(2+/3+), 50.0%, HPV-/p16(Neg/1+) and 31.6%, HPV+/p16(Neg/1+). HPV-/p16(Neg/1+) group had better 5-year RFS compared to the other groups: 10.7% versus 10.5% HPV+/p16(Neg/1+), 6.8%, HPV+/p16(2+/3+) and 3.3%, HPV-/p16(2+/3+). Besides adjusted HRs, HPV+/p16(2+/3+) group was significantly associated with an increased risk of death (HR = 2.3, 1.0 – 5.3). Although HPV+/p16(2+/3+) group had the greatest likelihood of disease recurrence but after adjusted HRs, it was no longer significantly associated with RFS. The median time to death was shorter in the

HPV+/p16(Neg/1+) group (20 months) compared to HPV-/ p16(Neg/1+) group (60 months). The median time to disease recurrence was shorter in the HPV+/ p16(Neg/1+) (28 months) compared to each of the other groups: HPV-/p16(Neg/1+) (53 months), HPV+/p16(2+/3+) (60 months) and HPV-/p16(2+/3+) (60 months).

OS was significantly better among those who were HPV16-/p16(2+/3+) group (log rank=22.133, $p$=0.000) and HPV16+/p16(2+/3+) for RFS (log rank=15.717, $p$=0.001), respectively (Figure 7.5A and 7.5B). HPV16-/p16(2+/3+) group had better 5-year OS compared to the other groups: 69.4% versus 42.4% HPV16+/p16(Neg/1+), 237.7%, HPV16/p16(Neg/1+). HPV16-/p16(2+/3+) group would not have been predicted in OS due to censored (n=12). HPV16+/p16(Neg/1+) had better 5-year RFS compared to the other groups: 20.0% versus 9.8% HPV16-/p16(Neg/1+), 8.3%, HPV16+/p16(2+/3+) and 4.8%, HPV16/p16(Neg/1+). Although HPV16-/p16(2+/3+) group had the greatest likelihood of death and disease recurrence but after adjusted HRs, it was only significantly associated with OS (HR = 1.9, 1.0 – 3.5). Unfortunately, the median time to death was unestimable. The median time to disease recurrence was shorter in the HPV16-/p16(Neg/1+) (39 months) compared to each of the other groups: HPV16+/p16(Neg/1+) (60 months), HPV16+/p16(2+/3+) (60 months) and HPV16-/p16(2+/3+) (60 months). Prognosis based on the individual biomarkers, HPV16 and p16 staining intensity, were compared to those based on the joint group effects. By jointly investigating with HPV16 status, the greater risk of death by p16 staining intensity among HPV16- group could be distinguished. Adjusted HRs revealed that older patients remained significantly associated a lower risk of death compared with the reference group. Adjusted HRs demonstrated that within tumour size, $T_2$ tumours appeared significantly associated with lower risk of death and disease recurrence

compared to reference group. Tumours of the alveolus seemed significantly associated with lower risk of death compared to pharynx tumours.

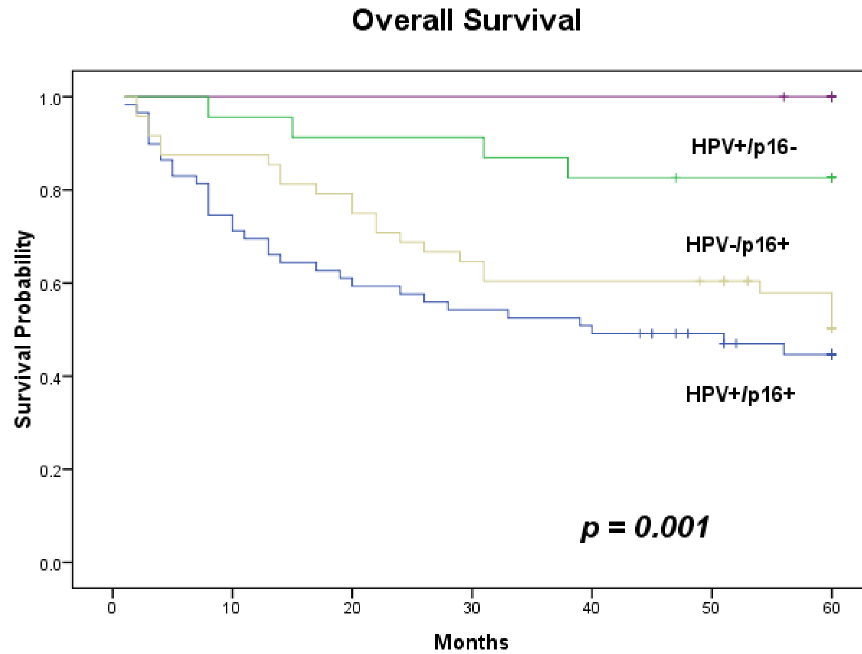



**Figure 7.2A: OS by HPV/p16 status as generated by Kaplan-Meier test.**
Vertical tick marks on curves indicate censored observations.

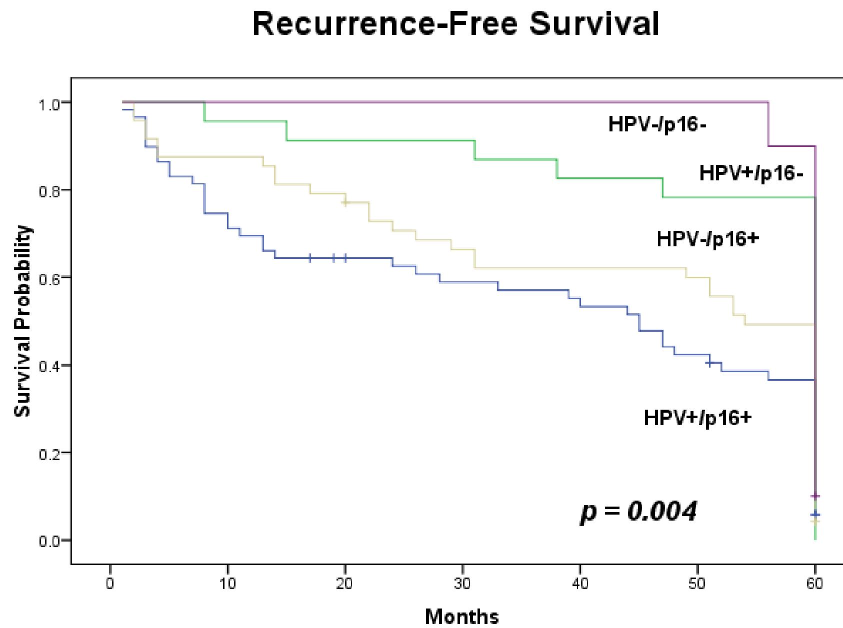



**Figure 7.2B: RFS by HPV/p16 status as generated by Kaplan-Meier test.**

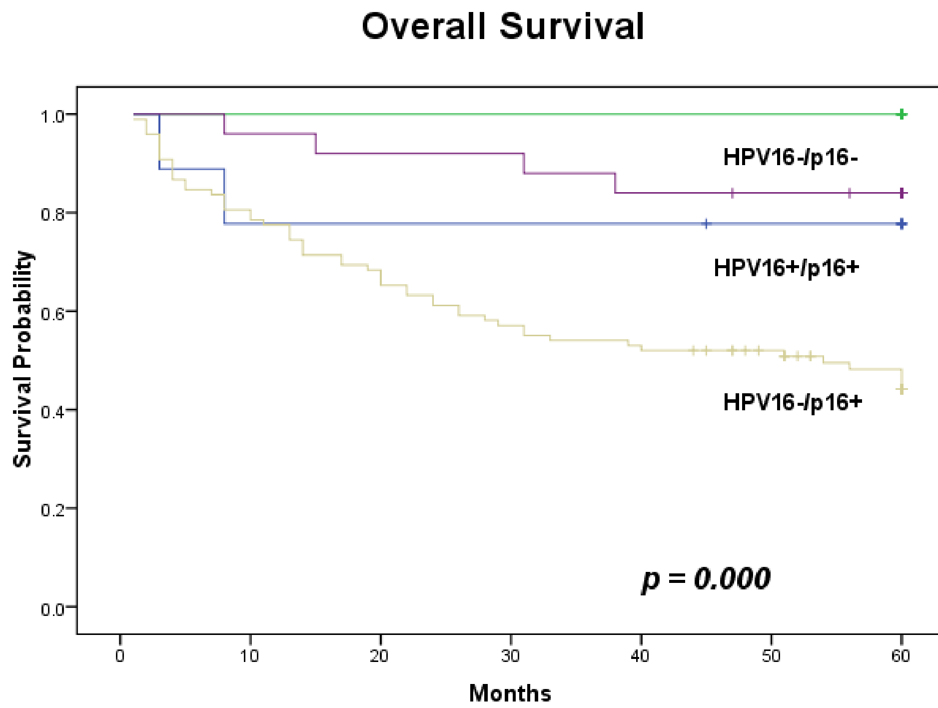



**Figure 7.3A: OS by HPV16/p16 status as generated by Kaplan-Meier test.**

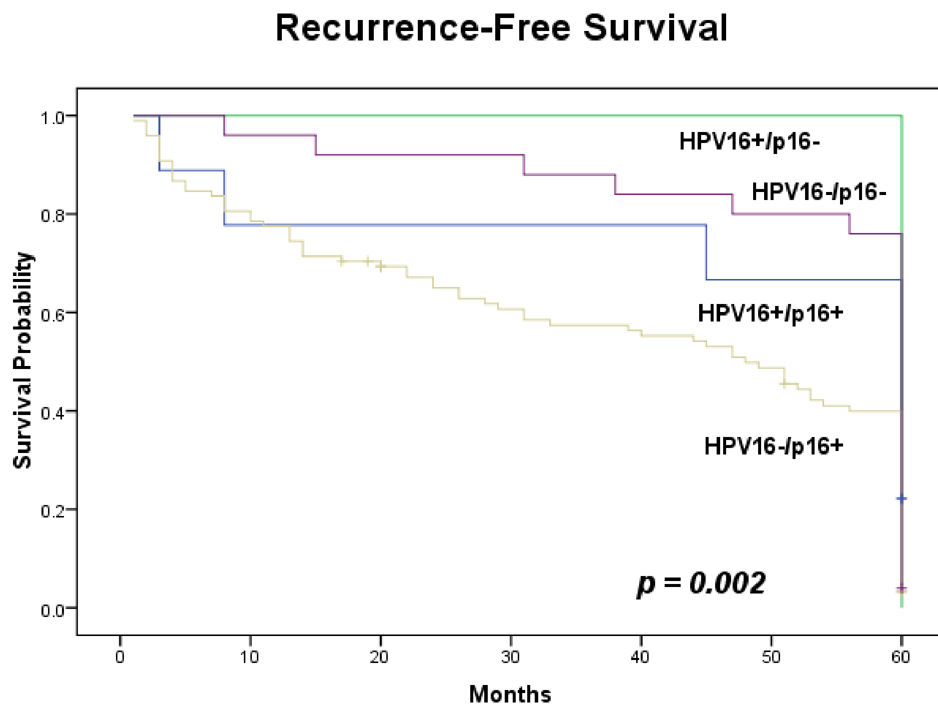



**Figure 7.3B: RFS by HPV16/p16 status as generated by Kaplan-Meier test.**

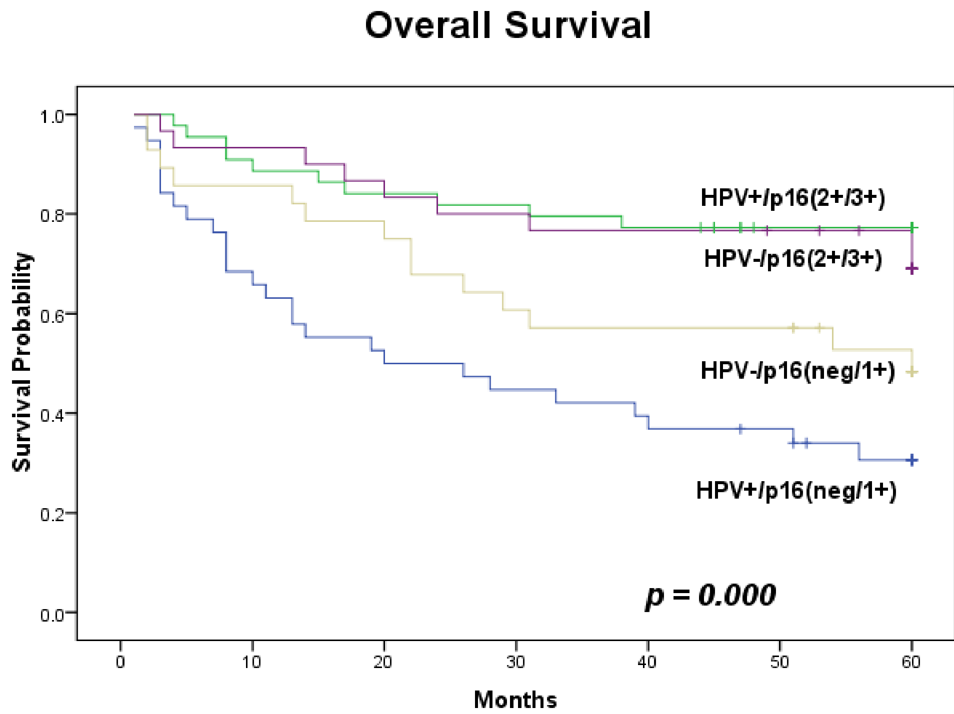



**Figure 7.4A: OS by HPV/p16 staining intensity as generated by Kaplan-Meier test.**

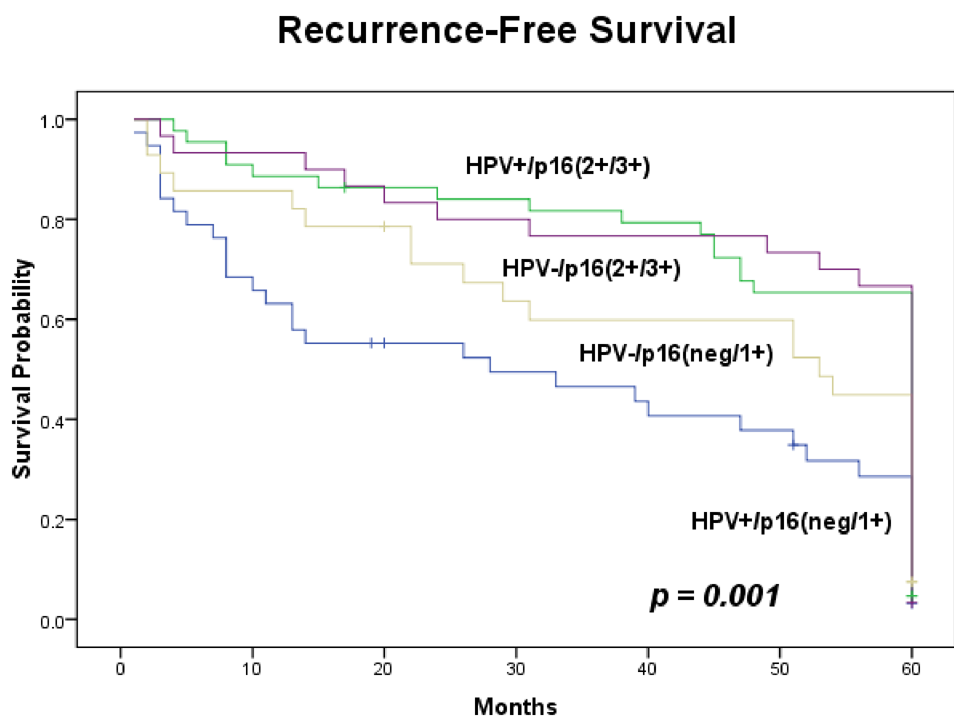



**Figure 7.4B: RFS by HPV/p16 staining intensity as generated by Kaplan-Meier test.**

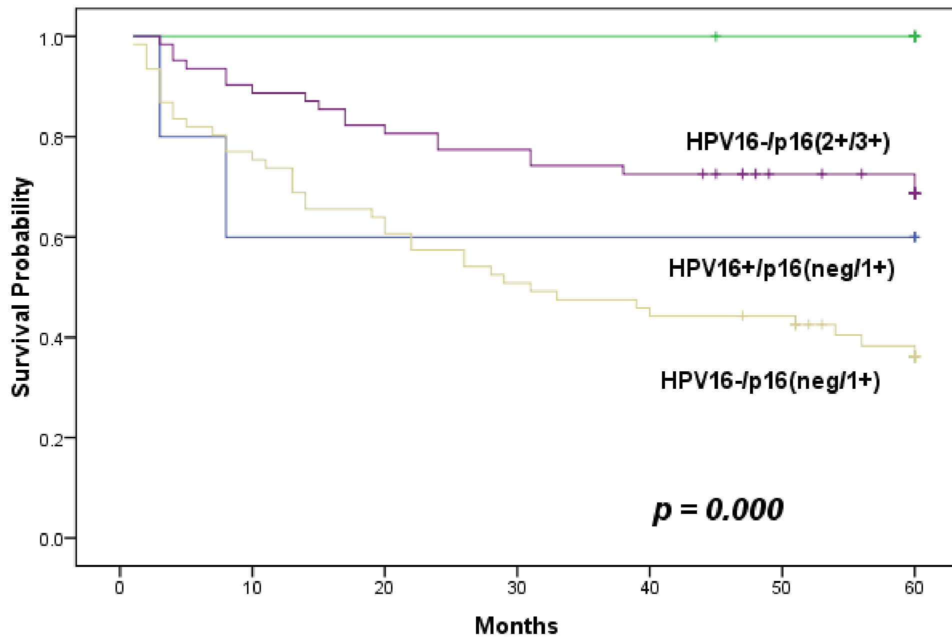



**Figure 7.5A: OS by HPV16/p16 staining intensity as generated by Kaplan-Meier test.**

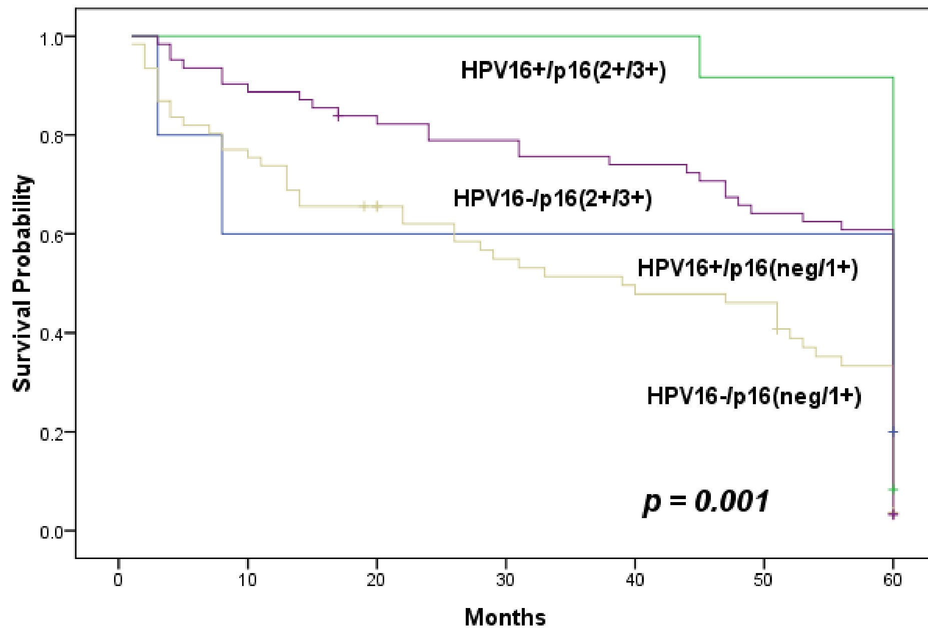



**Figure 7.5B: RFS by HPV16/p16 staining intensity as generated by Kaplan-Meier test.**

## 7.3 CONCLUSIONS

1. **The determination of prognostic significance risk factors on OS and RFS**

    - Kaplan-Meier survival analysis demonstrated that gender, age group, anatomic site, tumour size, regional lymph nodes, high risk HPV, HPV16, p16 positivity and p16 staining intensity were the potentially significant prognostic risk factors for patients OS.
    - Similarly, above potential prognostic risk factors were also found significantly associated with patients RFS except for age group factor.

2. **Univariate and multivariate analysis on OS and RFS using Cox regression model.**

    - In univariate analysis of total patients, high risk HPV, HPV16, p16 positivity, p16 staining intensity and HPV/p16 status predicted OS.
    - Nevertheless, the predictors for RFS were p16 positivity and p16 staining intensity according to univariate analyses.
    - In Cox multivariate analysis, adjusted HRs for gender, age group, anatomic site, tumour size and regional lymph nodes, p16 staining intensity was independently associated with OS.
    - Meanwhile, none of the potential independent predictors remained significant for RFS after adjusting for tumour size and regional lymph nodes based on Cox multivariate analysis.

**3. Cox proportional hazard models for prognostic value assessment.**

- After the inclusion of the following factors, gender, age group, tumour size, regional lymph nodes, survival and disease recurrence in the adjusted ORs, living patients was the only factor that remained significantly associated with p16 expression.
- With regard to tumours of the alveolus the risk remained significantly lowered and associated with p16 status despite adjustment in odds ratio compared to reference group. Nevertheless, for other anatomic sites the drop in odds ratio were not significantly associated with p16 status.
- Overall HPV and HPV16 status were significantly associated with p16 over-expression after adjustment for gender, age group, tumour size, regional lymph nodes, patient survival and disease recurrence.
- The associated covariates with OS based on multivariate analyses were gender, age group, tumour size and regional lymph nodes and should be included in developing appropriate models.
- The tumour size and regional lymph nodes were the inclusion factors in the final models since their significant association with clinical outcomes in RFS.

**4. The prognostic implications of HPV and p16**

- Individual analyses of significant prognosticators involving p16 and HPV status revealed that patients who exhibited p16-negative and p16 (neg/1+) had significantly better OS and RFS.
- In contrast, patients who did not exhibit HPV16 had significantly worse OS and RFS.
- Nevertheless, there was no significant difference in clinical outcomes for overall HPV cases in OS and RFS.

- Four combinations of HPV and p16 status (each with four subgroups) were generated for further clinical outcome comparison in association with patients OS and RFS.
- The first two were related to p16 positivity were as follows: HPV/p16 and HPV16/p16; the second two were related to p16 staining intensity.
- We found that OS and RFS were significantly worse among those who were HPV+/p16+ and this seemed consistent with individual analyses.
- The addition of HPV status revealed that the risk of disease recurrence was greater among patients who exhibited HPV+/p16-.
- The risk of death would not have been predicted by HPV/p16 status since the results were not estimable.
- Despite their contradictory results with the first two combinations above, OS and RFS with regard to HPV16/p16 status were significantly worse among patients who exhibited HPV16-/p16+. Thus, at this point the consistency with their previous separate analyses appeared to be well established.
- The combined HPV/p16 status analyses revealed that the risk of death and disease recurrence was lower among HPV16-/p16- group compared with reference group, HPV+/p16+.
- We demonstrated that 5-year OS and RFS were better in HPV16-/p16- and HPV+/p16+ groups, respectively.
- Thus far, with regard to p16 status, joint group effects satisfy that p16-negative was a better predictor for higher risk of disease recurrence compared to HPV tumours status.
- The similar patterns follow as for HPV status consideration in combined HPV/p16 status by p16 staining intensity.

- Generally, by taking into account p16 staining intensity into the combined assessment, p16(neg/1+) was a better predictor for lower risk of death and disease recurrence compared to tumour status.
- **The combined HPV/p16 status data demonstrates an association with survival and disease recurrence for head and neck cancer compared to each, HPV and p16 evaluated separately.**
- **Therefore, these findings could suggest that HPV and p16 status are independent predictors for patients risk of death and disease recurrence. They should be assessed in other larger studies to allow better prediction of prognosis to assist target HNC treatment.**
- Adjusted HRs revealed that older patients remained significantly associated in lower risk of death compared with reference group.
- Adjusted HRs demonstrated that within tumour size, $T_2$ tumours appeared significantly associated with lower risk of death and disease recurrence compared to reference group.
- Tumours of the alveolus seemed significantly associated with lower risk of death compared to tumours of the reference group.

# CHAPTER 8

# DISCUSSION

## 8.1 INTRODUCTION

There is a wealth of literature reporting the successful detection of HPV DNA in human cancers, especially studies on the role of HPV in cervical carcinomas. The proposal that HPV was involved in the aetiology of head and neck was reported as early as 1983 by (Syrjänen et al., 1983). Since then HPV has been recognised as a major aetiological factor in oral cancer (Schwart et al., 2001). Many head and neck studies have been published, but often the primary site is oropharyngeal rather than oral carcinoma. These studies have suggested that HPV positive oropharyneal cancers respond well to chemoradiotherapy and are associated with an improved prognosis. Therefore, knowing the HPV status for oropharyngeal carcinomas could impact on management and patient survival. In contrast, the primary treatment modality for oral cancer is surgery, so knowing the HPV status might not necessarily change the treatment but it might impact on prognosis. In many centres, p16 immunohistochemical staining is used as a surrogate marker for HPV for oropharyngeal cancer. However, it remains to be established whether p16 is a surrogate marker of the presence of HPV DNA in oral carcinoma. Despite the volume of literature on this topic, methodological differences between studies make comparisons of results difficult and any conclusions have to be interpreted with this caveat in mind.

It is important to consider that HPV infection involves highly complex mechanisms which are still poorly understood. There are various detection methods and the majority of them focus on the prime targets of HPV in their sampling, i.e. keratinocytes (Galan-Sanchez and Rodriguez-Iglesias, 2010 and Snidjer et al., 2010). A few controversial issues still underline the details of the mechanism of the HPV-host interaction (Letian and Tianyu, 2010; Horvath et al., 2010; Pittayakhajonwut and Angeletti, 2010; Syrjänen, 2010; Chow et al., 2010; Pim and Bank, 2010 and Steinberg and Norrild, 2010). There is no doubt that the holistic understanding of the significant role of HPV in oral carcinogenesis might be exploited for its diagnosis, prognosis and treatment.

As a routine test, it would be advantageous to develop screening tools for a larger population which would not have a significant economic impact and would therefore be more acceptable to the government. P16 imunohistochemistry seems relatively cheaper when compared to HPV genotyping. Many studies on head and neck cancer have utilised p16 as a surrogate marker for HPV DNA (Li et al., 2001; Klussmann et al., 2003; Wittekindt et al., 2005; Begum et al., 2005; König et al., 2007; Hafkamp et al., 2008; Smith et al., 2008; Smith et al., 2010 and Singhi and Westra, 2010). Yet, the evidence for such a use for p16 in oral cancer is somewhat limited, unconvincing and, in some cases, controversial (Nemes et al., 2008; Greer Jr. et al., 2008 and Friedrich et al., 2010). Nevertheless, in the majority of cases, the presence of HPV would not change the management of oral carcinoma, which is primarily surgical, as opposed to radiotherapy as is the case with oropharyngeal carcinoma. The translational potential for this work could be to identify patients with improved prognosis or potentially it could be used to inform patient management.

A few challenging questions have provided the motivation to embark upon this investigation. Would the finding of a high incidence of high risk HPV provide support for a vaccination programme for at risk groups, if indeed we could identify such a group of patients? This is an important issue and has already had an influence at a political level with the introduction of a vaccination programme for those at risk of cervical carcinoma. We found that 15% of our head and neck (predominantly oral) cancer cases were high-risk HPV positive. This would suggest that HPV may only be involved in a small subset of cases. So, to answer the question about vaccination, how do we identify those at risk? The subsequent question that is quite relevant, is how does HPV have an impact on prognosis, is HPV positivity associated with improved prognosis in oral cancer? If vaccination was offered to a group of patients at risk of oral carcinoma, lacking the traditional risk factor of smoking, do these carcinomas behave differently? Unfortunately, the data for smoking was only available for the cases in the pilot study, which was prospectively collected, but not for the larger retrospective study cohort. Another area of concern relates to the survival data. The death certificates were requested for the carcinoma cases but these often gave disseminated cancer as a cause of death, but this was determined without reference to a post mortem examination. We therefore do not really know whether the data is disease specific. Ultimately, our sample is relatively small in comparison to studies on cervical and other cancers. These issues are among the major challenges of our studies.

## 8.2 HPV PREVALENCE IN HEAD AND NECK CANCER

Generally tissue specimens are available as FFPETs. Most studies reported that there are problems related to difficulties in the extraction of nucleic acids from FFPETs, especially related to low DNA yield due to DNA degradation and poor DNA quality (Coombs et al., 1999; Wickham et al., 2000; Sato et al., 2001; Kim and Chae, 2001; Man et al., 2001; Wu et al., 2002 and Simonato et al., 2007). It was important to optimise the DNA extraction during the pilot study in order that our optimised protocol could be utilised in the larger cohort of tissue i.e. the oral disease progression study. There was concern about the possible sources of contamination that might have jeopardised our findings. The implementation of a standard operating procedure and very strict measures in relation to sample handling, gave us the greatest quality assurance of the specimens for this study. As an investigator, the precautions undertaken in the laboratory were to irradicate any source of contamination.

For the pilot study, we utilised various PCR-based techniques including conventional PCR, $SPF_{10}$-$LiPA_{25}$ (version 1) and PM-PCR RHA. In terms of cost, the first method was relatively cheap compared to the latter two techniques. The rational behind attempting various PCR-based approaches was to compare the methodologies to determine which one was better to use for the larger cohort. Analogically, we used a "single bullet for at least two targets". First and foremost, we gained invaluable experience dealing with the various protocols. Secondly, we benefited from the results generated by each technique in that we could verify the results using the simplest technique by the other two. In that way we could be sure that the results of our pilot study were accurate and provided information on HPV prevalence and evidence of

multiple HPV infections. Due to budget constraints we were unable to utilise all three methods in the larger cohort. Upon completion of the pilot study, the conventional PCR method was selected, as it had been shown to be reliable. One of the set backs of this option is that we were unable to identify multiple HPV infections in the larger cohort.

The prevalence of all HPV types detected in the pilot study (n=60) was 67%, but by increasing the sample size (n=140) the overall HPV positivity was 59%. Another oral study, which had a similar methodology to ours, reported that the prevalence of HPV in 15 oral carcinomas as 60% and all of them HR-HPV (Zarei et al., 2007). In this study, HPV16 was detected in a smaller proportion of the sample in that only 8 of the 60 cases were HPV16 positive but ours was a much larger study. Even in our combined carcinoma cohort of 140 samples the actual number of cases that were HPV16 was 17. The overall HPV prevalence was not statistically significant different across pathological groups. In the benign lesions (n=84), dysplasias (n=12) and carcinomas (n=80) the percentage of HPV-positive were 51%, 42% and 53%, respectively. There were no normal tissues used as a control group in this study. However, the reported HPV incidence in normal oral mucosa varies from as low as 0% (Bouda et al., 2000; ÓFlatharta et al., 2003, Gonzalez et al., 2007) to as high as 36-55% (Sugiyama et al., 2003; Giovannelli et al., 2006 and Zhang et al., 2004).

We performed a literature search for studies carried out on the prevalence of HPV in oral mucosal lesions and carcinomas over the period of ten years between 2000 and 2010, We restricted the outputs of our searches with other preset selection criteria such as the type of samples, DNA extraction methods and HPV detection techniques used which were similar to the current study. Only two studies were identified that reported

HPV16 prevalence in oral dysplasia using detection methods. Both studies reported that more than a 60% HPV16 positivity (Patiman et al., 2001 and Sugiyama et al., 2003). In fact, the differences in the sample size might not affect the overall percentage statistically in both studies (*n*=30 and *n*=51) in respect to the current study. We treated our findings in the dysplasia cohort with caution as our sample size was relatively small. As for the benign lesions, there have been reports of as high as 91% positivity (Gonzalez et al., 2007). However, the sample size was very small (n=11), so this result might not reflect the actual HPV prevalence. If we consider the other three studies that are relatively comparable in their sample size (n=35-40), they reported the incidence of HPV ranging from 26-55% (ÓFlatharta et al., 2003, Llamas-Martinez et al., 2008 and Jimenez et al., 2001). Our results suggest that our findings are comparable even though our sample was more than double the size of those studies. Fourteen studies on HPV prevalence in head and neck carcinomas were successfully retrieved from the databases, only ten of them clearly identified themselves as studies related to oral carcinomas. A large variation of HPV prevalence was reported within these ten studies ranging from 8% (Kansky et al., 2003) to as high as 92% (Patiman et al., 2001). Therefore, we pooled the data of ten studies (n=496) to obtain an average prevalence of HPV in oral carcinomas which was 40%. These results emerged not to be significantly different from our findings.

We categorised our results from the pilot study based on specific HPV types into three major groups including HR-HPV, LR-HPV and β-HPV types. The last group was not applicable for respective comparison with the larger cohort or oral disease progression since β-HPV genotyping was only undertaken in the pilot study. We found 18% HR-HPV, 57% LR-HPV and 18% β-HPV types in our pilot study samples. In the

pooled data cohort, we found the percentage of HR-HPV increased to 25% but was not significantly different for LR-HPV at 59%. The oral disease progression study demonstrated that in oral benign lesions, 14% were HR-HPV and 31% were LR-HPV; in dysplasias, 8% were HR-HPV and 17% were LR-HPV and in carcinoma, 16% were HR-HPV and 30% were LR-HPV. From the results of the oral disease progression study, we observed no significant difference in the prevalence of either HR-HPV or LR-HPV but the ratio based on percentage between HR-HPV to LR-HPV was approximately 1:2 across each pathological group. Our results were in contrast to Miller and Johnstone, (2001) who estimated that HR-HPV was approximately 3 times more frequent than LR-HPV in carcinomas (Miller and Johnstone, 2001). Generally, within carcinomas, the percentage of LR-HPV prevalent in the pilot study, pooled data or oral disease progression study was consistent with a range of 57-59%. In oral benign lesions, other studies reported that HR-HPV was detected at 30% (Gonzalez et al., 2007) and 40% (Llamas-Martinez et al., 2008). It seems that both results are relatively consistent with our current findings. The latter study specifically stated that the sole contributor to the total percentage was HPV16. We were unable to make appropriate comparisons since the authors did not provide the actual percentage of HR-HPV in their cohorts (Patiman et al., 2001 and Sugiyama et al., 2003). Within carcinomas, three studies reported a slightly higher percentage of HR-HPV prevalence compared to the current study, 33% (Llamas-Martinez et al., 2008), 42% (Chang et al., 2003) and 52% (Gonzalez et al., 2007). None of the reports related to LR-HPV prevalence in oral benign lesions and oral dysplastic tissues, but one study did report data for carcinomas (Kansky et al., 2006). Unfortunately, the total prevalence reported was presented as the pooled data of HPV6 and HPV16 positivity. Two out of forty HPV types in our pilot study were not identifiable for their type specificity by using the current $SPF_{10}$-$LiPA_{25}$

(version 1) method. This may suggest that these may potentially emerge to be novel HPV types.

We are not the first group to report the presence of β-HPV in oral mucosal lesions. Earlier, de Villier and colleagues reported the presence of seven novel HPV types of which six were β-HPV types comprising HPV type 98, 99, 100, 104, 105, and 113 and one α-HPV type, HPV94 (de Villier et al., 2009). We referred to the earlier publication of the same investigators who had reported that HPV types 15, 23, 36, 38, 76, 80 and 93 as we found in the current study, were identified as β-HPV types and these are more commonly found in human cutaneous lesions (de Villier et al., 2004). de Koning et al. (2007) later confirmed that all seven β-HPV types of our current findings were actually persistently present in plucked eyebrow hairs of healthy individuals (de Koning et al., 2007). Based on our strict measures and the precautions taken in sample handling, we are fully confident that these are true findings and very unlikely to be contamination. Unfortunately, the actual role played by those HPV types in oral mucosa remains unresolved. To the best of our knowledge, there are no previous reports of the actual prevalence of β-HPV in oral mucosal lesions. This may be an area for future research to determine whether β-HPV plays an active role in oral carcinoma. Pfister (2003) reported that HPV8 and HPV5 had a significant role in which both were classified as 'high-risk' among β-HPV types (Pfister, 2003). In addition, it was believed that those β-HPV types were associated with patients who had been diagnosed with the rare hereditary disease, epidermodysplasia verruciformis. Three out of eleven β-HPV types in our pilot study were not identifiable for their type specific by using current PM-PCR RHA method and these may emerge as the novel HPV types.

We investigated the possibility that there were multiple HPV infections in our pilot study cohorts. We observed that within positively detected HPV, 68% exhibited single type infection, 25% double types of HPV and 8% multiple HPV infections. Terai and colleagues (1999) reported that the prevalence of multiple HPV infections in normal oral cavity was 56% of 30 adults (Terai et al., 1999). In our study HPV6 was the only LR-HPV detected, the discussion related to LR-HPV refers to HPV6. In single infections, LR-HPV demonstrated the highest proportion at 35%, 3% for HR-HPV (HPV16 and HPV18) and 7% infection related to β-HPV types. In double infections, co-infection of HPV6 and HPV16 was detected at 8.3% and 13% was related to co-existing of HPV6 with other β-HPV types and 3% involving HPV6 with HPV18 infections. One problem encountered was that we were unable to determine which HPV type was the dominant type possibly playing a significant role in the aetiology of cancer. A number of studies suggested that viral load assessment could address this issue. Seven studies attempted to quantify viral load of HPV in cervical specimens by utilising hybrid Capture II (Ferreira Santos et al., 2003), real-time PCR (Swan et al., 1999; Flores-Munguia et al., 2004; Guo et al., 2007; Fontaine et al., 2008; Marks et al., 2009; and Tadokoro et al., 2009) or a combination of real-time PCR and PCR-EIA (Hesselink et al., 2005). Klussmann and colleagues (2003) relied on nested PCR for viral load estimation in tonsillar carcinomas (Klussmann et al., 2003). Three studies suggested the utilisation of PCR-Enzyme-Linked Immunosorbent Assay (PCR-ELISA) in oral or oropharyngeal studies (Kreimer et al., 2005), a combination of multiplex PCR and in situ hybridisation (D'Souza et al., 2007; Gillison et al., 2008). Another four studies reviewed the effectiveness of this approach in assessing the predominant HPV type (Chaudhary et al., 2009; Goon et al., 2009 and Salem, 2009) and one in cervical cancer (Lillo and Uberti-Foppa, 2006). Two untypable α-HPVs (both were present in

combination with another HPV type) and three untypable β-HPVs (two were detected in postulated single HPV infection and one as multiple HPV infection with α-HPV types) could also be novel HPV types. Since conventional PCR, PM-PCR-RHA and $SFP_{10}$-$LiPA_{25}$ (version 1) approaches failed to identify them in our pilot study, other methods should be employed for further HPV genotyping purposes.

In the combined carcinoma group, we observed that HPV16 was more frequently encountered in females compared to males, and HPV6 was common in older patients. There is a postulated relationship between HR-HPV and past sexual history which could possibly explain the gender difference, but this aspect was out-with the scope of this study (Dahlstrom et al., 2011). In addition, HPV16 was frequently identified in patients who survived. The overall HPV prevalence was not significantly different across anatomic site and tumour size but HPV6 was found at higher proportion in cases with lymph node metastasis compared to the rest of HPV types concerned. To the best of our knowledge thus far, we are unable to provide any satisfactory comparison from other related studies on the relatively high prevalence of low risk HPV in our study.

## 8.3 p16 EXPRESSION IN HEAD AND NECK CANCER

In the carcinomas of the pilot study 78% were p16 positive and in the combined carcinomas group it was 76%. There were no normal tissues used as a control group in this study. However, the reported p16 expression in normal oral mucosa varies from 0% (Angiero et al., 2008 and Buajeeb et al., 2009), 1.45% (Angiero et al., 2009) to as high as 23% (Bilde et al., 2009). Within pathological groups the percentage of p16 positivity

increased from 51% in oral benign lesions, to 67% in dysplasias and then 75% in carcinomas. Shintani et al. (2002) reported that the percentage of p16 expression in the oral cavity was 100% in normal tissues normal (n=20), 88.1% in dysplasia (n=42) and 30.8% in carcinomas (n=117). These results seem to contradict our current findings, but we could argue the discrepancies observed could be attributed to differences in sample size and the scoring scheme used for p16 assessment. Two studies reported a significantly lower percentage of p16 positivity, 43-58% compared to our study in benign oral lesions (Cao et al., 2010 and Fregonesi et al., 2003). Six studies on p16 expression in oral dysplastic tissues reported that the percentage varied from 0-90% (Buajeeb et al., 2009; Bilde et al., 2009; Cunningham et al., 2006; Cao et al., 2010; Bradley et al., 2006; Angiero et al., 2008 and Fregonesi et al., 2003). The pooled data of these six studies (n=258) suggested an average percentage of p16 positivity of 34% (approximately 50% lower than our study). In fact, we could argue that the number of samples in our dysplasic group (*n*=12) may not reflect the actual percentage of p16 expression in this pathological group. We also found seven studies investigating the expression of p16 in oral carcinomas using immunohistochemistry and found that the percentage of p16 positivity varied from 13-94% (Muirhead et al., 2006; Buajeeb et al., 2009; Weinberger et al., 2004; Suzuki et al., 2006; Bilde et al., 2009; Angiero et al., 2008 and Fregonesi, et al., 2003). We pooled the data of seven studies (n=326) and calculated that the average percentage of p16 expression in oral carcinomas was 27%.

There was a decreasing pattern of p16 expression across pathological groups based on an average percentage of the pooled data demonstrated by Shintani et al., (2002), but the percentage of p16 positivity of dysplastic and carcinomas appeared to be comparable. A summary of the published data on p16 staining for each pathological site

is as follows: oral benign lesions from 43 to 58% positive (two studies), dysplasias were 0-90% positive, (six studies) and carcinomas 13-94% positive (seven studies). It was difficult to make a generalisation on p16 expression across pathological groups mainly related to dysplasias and carcinomas as both exhibited larger variations in p16 expression. However, in benign groups, the percentage of p16 expression observed in the present study seemed within the range.

In the pilot study, p16 status (positivity/staining intensity) was significantly associated with male gender, older patients, $T_3$ tumour size, $N_2$ nodal metastasis and disease recurrence. Similarly, in the combined carcinomas cohort significant association was observed but only for the male gender and the older patients by p16 status. Within this larger cohort, p16 status was significantly associated with $T_1$ tumour size and patient survival. In addition, p16 positivity was significantly different in anatomic site (alveolus and lip). An in depth comparison within this cohort found that p16 status was significantly higher in males, older age patients, tumour size $T_3$ and $T_{2,}$ and patients who did not survive. Generally in oral benign lesions and dysplastic tissues the p16 status was not significantly associated with gender, age group, anatomic site, patient disease outcome and status. However, in carcinomas the p16 status was significantly higher in males, older patients and patients who had not survived, tumours of the tongue and floor of the mouth compared to soft palate, tumour size, $T_3$ and $T_2$ compared to $T_1$ and in well and poorly differentiated tissues compared with moderately differentiated tissues. Yuen et al. (2002), on the other hand, did not find a significant correlation between p16 expression and gender, age group, disease recurrence or survival (Yuen et al., 2002). However, our results were in agreement with Yuen et al. (2002) that no significant association was observed between p16 expression and nodal metastasis. Our

results appeared in accordance with Muirhead et al. (2006) in that no significant correlation was observed between p16 expression, tumour stage and patient's survival.

## 8.4 CORRELATION AMONG DEMOGRAPHIC AND CLINICOPATHOLOGICAL PARAMETERS, HPV AND p16 EXPRESSION

The pilot study and the pooled carcinoma cohort produced similar results in that no association was observed between HPV status and disease recurrence. No direct comparison could be made between both cohorts for some of the parameters such as smoking and alcohol, since the data were not available for the larger cohort. Earlier, we reported a significant association between HPV status and smokers based on our pilot study results. These results are consistent with the findings of Reimer et al., (2007). In the pilot study, no significant association was observed between HPV status and clinicopathological parameters for tumour size, nodal metastasis, age group and gender but this was not the case for the pooled carcinomas cohort. In this larger cohort, overall HPV and LR-HPV status were associated with patients who had tumour more than 2 cm in size and nodal metastasis. HR-HPV or specific to HPV16 status were associated with patient survival who had a tumour of less than 2-cm. In addition, α-HPV, LR-HPV and HPV16 were associated with nodal metastasis, older patients and female gender, respectively. Generally, across pathological groups, from benign to carcinoma, we found an increase in the significance of the association between HPV status and demographic and clinicopathological parameters. Therefore, we could suggest that HPV status was associated with oral disease progression. Regardless of the pathological group, HR-HPV appeared not to have any association with clinical parameters.

However, studies done on oral leukoplakia patients did not find any association between HPV status and malignant transformation (Yang et al., 2009).

Similar results were found in the pilot study and the combined carcinomas cohort with regard to p16 status (p16 positivity and p16 staining intensity) which was not associated with disease recurrence or nodal metastasis. In addition, p16 staining intensity was negatively associated with patient survival and was observed in both cohorts. However, both cohorts differed in that a significant association was only observed in the combined carcinoma cohort between p16 status and male gender, older age patients, tumours of the alveolus and tumour size more than 2 cm. No significant association was found between p16 expression and clinical parameters in oral benign lesions and dysplastic tissues. However in the combined carcinoma group, p16 status was significantly associated with male gender, older age patients, tumours of the floor of the mouth and the tongue, patient survival, tumour size, $T_2$ and $T_3$. In addition, p16 positivity was significantly associated with histological grade and this finding is consistent with that of another study (Reimers et al., 2007).

In the pilot study, we found that results form Kappa Cohen's and Chi-square demonstrated that overall HPV status and p16 expression was not significantly associated. However, p16 staining intensity was significantly associated with HPV status specifically HR-HPV and HPV16 and these findings appeared consistent with another related study (Kong et al., 2009). The results from the larger combined carcinoma cohort further confirmed this association in that HPV16 was inversely associated with p16 status (positivity and staining intensity). A recent meta-analysis involving 34 studies to include 5681 patients with HNSCC reported that the prevalence

of overall HPV and HPV16 were 22% and 19%, respectively (Dayyani et al., 2010). They also concluded that the overall HPV status was correlated with p16 expression and HPV16 was the predominant contributor for these associations, 87%.

Smith et al. (2008) investigated the association between HPV and p16 status in oropharyngeal cancers (n=301) reported that positive concordance (HR-HPV+/p16+) and negative concordance (HPV-/p16-) were 21% and 59%, respectively (Smith et al., 2008). Smeets et al. (2007) investigated a smaller cohort for association between HPV16 and p16 status in unspecified head and neck cancers from FFPETs (n=48) reported that positive concordance (HPV16+/p16+) and negative concordance (HPV16-/p16-) were 31% and 44%, respectively (Smeets et al., 2007). Comparing results from the former study with our current pooled carcinomas results (n=140), we found that positive concordance (HR-HPV+/p16+) and negative concordance (HPV-/p16-) were 17% and 42%, respectively. For comparison with the latter study, our current study results were positive concordance (HPV16+/p16+) and negative concordance (HPV16-/p16-) were 6% and 18%, respectively. Differences with our results could be attributed to the different sample sizes and also additional contributing factors such as the site specific proportion of the samples, HPV detection system and p16 expression methodology used.

Our results disagreed with other studies that concluded HPV was positively associated with p16 expression. We found twelve highly related studies in terms of methodologies with our study which reported an association between HPV and p16 expression in various sites: oral dysplasia (Cunningham et al., 2006); oropharyngeal carcinoma (Kumar et al., 2008, Smith et al., 2008; Nichols et al., 2009 and Al-Swiahb et

al., 2010); tonsillar carcinoma (Klussmann et al., 2003, Li et al., 2004, Begum et al., 2005 and Hafkamp et al., 2008); head and neck carcinoma (Smith et al., 2008 and Smith et al., 2010) and cervical carcinoma (Lambert et al., 2006). Other studies also reported similar conclusions about the HPV/p16 association, but these studies differed from our study in that they utilised in situ hybridisation for HPV detection. These twelve studies are as the follows: OSCC (Fregonessi et al., 2003), head and neck carcinoma (König et al., 2007 and Singhi and Westra, 2010) and cervical carcinoma (Sano et al., 1998; Sano et al., 2002; Tsai et al., 2005 and Lakshmi et al., 2009). On the other hand, five studies did not find any association between HPV and p16 expression (Greer Jr. et al., 2008; Nemes et al., 2006; Cao et al., 2010; Samama et al., 2006 and Galmiche et al., 2006). Despite the conflicting reports of the correlation between HPV and p16, our findings thus far suggest that p16 could be potentially acts as a surrogate marker for high risk HPV.

## 8.5 RISK ASSESSMENT WITH DEMOGRAPHIC AND CLINICOPATHOLOGICAL PARAMETERS ASSOCIATED WITH HPV AND p16 STATUS

In the pilot study, we demonstrated that smokers, patients who were still alive and those with disease recurrence were significantly associated with an elevated risk by HPV status compared with each with reference group (non-smokers, dead and recurrence-free patients, respectively). The present investigation's result on smoking was consistent with Dayyani et al. (2010), OR=4.0, 95%CI (1.3-12.0) and OR=3.53, 95% (2.69-4.64), respectively. We observed that in the pilot study, the pooled carcinomas cohort and across pathological groups, all demographic and clinicopathological parameters concerned including gender, age group and anatomic site were not significantly

associated with risk by overall HPV status. In addition, across pathological groups, patient survival and patient with disease recurrence were not significantly associated with risk by overall HPV status. Similarly, in dysplastic tissues, the above mentioned parameters were not associated with risk by HR-HPV status. In oral benign lesions and carcinomas, female gender and younger patients were significantly associated with elevated risk by HR-HPV status. Tumours of the buccal mucosa were significantly associated with elevated risk compared with the tumours of the floor of the mouth and tonsil in benign groups by HR-HPV status. In carcinomas, patient that were still alive with disease recurrence, had tumour size $T_2$ and $T_3$, nodal metastasis $N_1$, poorly and basaloid differentiated tissue were significantly associated lower risk by HR-HPV status. Smith and colleagues, (2007) reported that antibodies against HPV16 E6 or E7 were correlated with risk of nodal status (Smith et al., 2007).

In the pilot study and pooled carcinomas cohort, male gender, older patients who had $T_2$ and $T_3$ tumour size, nodal metastasis, $N_0$, $N_1$ and $N_2$ and disease recurrence were significantly associated with an increase risk by p16 status. No significant association was observed in the combined carcinomas cohort for anatomic site and tissue differentiation grade with risk by p16 status. In this cohort, the caveats to our findings were the sample size and the fact that sub-site analysis could not be undertaken. We observed that across pathological groups, all demographic and clinicopathological parameters concerned were not significantly associated with risk by p16 status except in the carcinomas group for patients with disease recurrence and nodal metastasis. In the combined carcinoma group, older patients remained significantly associated with a good prognosis after adjusted HRs. Tumour size $T_2$ appeared significantly associated with a good prognosis and non-recurrence.

## 8.6 SURVIVAL ANALYSIS AND PROGNOSTIC IMPLICATIONS OF HPV AND p16

In the pilot study, a survival analysis suggested that tumours of the pharynx, lower lip, tonsil and buccal mucosa and tumour size, $T_3$ could be independent prognosticators for poor patient overall survival (OS). No association was found for HPV and p16 status or combined HPV/p16 status. In the combined carcinoma study, the survival analysis demonstrated that female gender, younger age patients, tumours of the soft palate, tumour size, $T_1$, negative nodal metastasis, HR-HPV-positive, HPV16-positive, p16-negative and p16 (neg/1+) were the potentially significant prognostic factors for patients OS. Similarly, the above mentioned risk factors were also found significantly associated with patients RFS except for age group. The associated covariates with OS based on multivariate analyses using Cox regression model were gender, age group, tumour size and regional lymph nodes and should be included in developing appropriate risk models. The tumour size and nodal metastasis were the inclusion factors in the final models since they were significantly associated with clinical outcomes in RFS. We found that overall HPV and HPV16 status were significantly associated with p16 over-expression after adjustment for gender, age group, tumour size, nodal metastasis, patient survival and disease recurrence. Smith and colleagues (2008) reported that p16 expression was 35%, and the prevalence of HR-HPV was 27% from 301 paraffin-embedded tissues (Smith et al., 2008). More than fifty five percent of their samples were from the oral cavity. Two major differences were found between this study and our work in relation to the HPV detection method used, and the semi-quantitative scale used for p16 expression scoring. They utilised a dot blot hybridisation technique together with PCR amplification for HPV detection and applied unspecified percentage,

but the borderline for p16 positivity was lower than 10% positive staining whereas for the present study we set 10% as the cut of point for p16 positivity. We suggest that the differences in the sample proportion together with the chosen methods for HPV and p16 expression contributed to the discrepancies in the results.

Individual analyses of significant prognosticators involving p16 and HPV status revealed that patients who exhibited p16-negative and p16 (neg/1+) had significantly better OS and RFS. Our results were not in agreement with those of Smith et al. (2008). However, HPV16 positive patients had a significantly better OS and RFS in agreement with Smith et al. (2008) but we treated our findings with caution since the latter study did not specifically refer to HPV16 but the pooled data of HR-HPV. Dayyani et al. (2010) later reported in a meta-analysis of 34 studies on 5681 HNSCC patients that HPV positive patients were associated with a significantly better survival (Dayyani et al., 2010). They reported that there was an improved OS by HPV status as (HR=0.42, 95% CI=0.27-0.56, $p<0.0001$) compared to our present findings (HR=0.40, 95% CI=0.16-1.0, $p<0.05$). OS and RFS with regard to HPV16/p16 status were significantly worse among patients who exhibited HPV16-/p16+. The combined HPV16/p16 status data demonstrated an association with survival and disease recurrence for head and neck cancer compared with HPV16 and p16 in isolation. In agreement with the suggestion made by Smith et al. (2008) that HPV and p16 should be accessed together for better predict prognosis (Smith et al., 2008). Therefore, our findings could suggest that HPV16 and p16 status are the independent predictors for patient risk of death and disease recurrence and may improve the prediction of prognosis which may also benefit in the targeted management of the head and neck cancer.

# CHAPTER 9

# CONCLUSIONS

The prevalence of all HPV types detected in carcinomas was 67% for the pilot study of which 18% were HR-HPV and 59% for the combined carcinoma sample of which 25% were HR-HPV. The majority of HR-HPV was HPV16. In addition, the HPV prevalence was 51%, 42% and 53% for the benign lesions, the dysplasias and carcinoma respectively in the disease progression study. More specifically, the prevalence of HR-α HPV was 14% in benign lesions, 8% in dysplasias and 16% in carcinomas. The similar prevalence of HR-HPV in benign and malignant lesions raises some question over the role of HR-HPV in oral carcinoma. This similarity might be a function of the sample size. In the combined carcinoma cohort the proportion of HR-HPV was greater. This may suggest that the study may have been insufficiently powered. Future work should be based on larger sample sizes in order to determine whether there is a subset of HNSCC where HR-HPV has an important possible causal role.

We demonstrated the presence of multiple HPV types in head and neck tissue both low risk and high risk in the same tissue and in some cases alpha and beta HPV. However, we were unable to determine which genotype was the dominant one. Future work investigating viral load could address this issue The finding of β-HPV types in our mucosal specimen is a relatively novel finding and the actual role played by the cutaneous type in head and neck mucosa merits further investigation.

Analysis of the combined carcinoma data showed an association between HPV status and gender, age group, survival, nodal metastasis and $T_3$ tumour size. HPV16 was predominantly present in female patients. This finding of a gender difference for HR-HPV may suggest a relationship between HR-HPV and past sexual history. However, this issue of sexual history was beyond the scope of this study. No association was found between overall HPV status and survival or disease recurrence. However, HPV16 was associated with improved overall survival and recurrence-free survival. In the pilot study where we had data on other aetiological factors we found that HPV status was positively associated with smoking.

p16 positivity varied from 76-78% in carcinomas and the p16 positivity increased proportionally by a comparison of benign lesions with dysplasias and carcinomas. This is in contrast to the findings of HPV overall and HR-HPV in a comparison of benign with malignant tissues. p16 status (p16 positivity and p16 staining intensity) was not associated with disease recurrence and positive nodal disease. In addition, positive p16 staining and high staining intensity was associated with a poorer overall survival. In carcinomas, p16 status was significantly associated with male gender, an older age group, anatomic site (alveolus and lower lip), and $T_2$ tumour size. In addition, p16 positivity was significantly associated with histological grade.

We found that overall HPV status was not correlated with p16 expression. Nevertheless, p16 staining intensity was significantly associated with HPV status specifically HR-HPV and HPV16. However, a correlation was found between p16 and HPV16 which may suggest that p16 could potentially act as a surrogate marker for high risk HPV. However, the lack of concordance between HPV and p16 would suggest that in

isolation p16 may not be a reliable marker for HR-HPV and should not be relied upon in isolation in predominantly oral carcinoma. Interestingly, a better overall survival was found with carcinomas that were HPV16 negative and p16 negative. Our findings could suggest that HPV16 and p16 status may be independent predictors for prognosis and disease recurrence.

Future work to compare HPV and p16 on specifically oral carcinoma with oropharyngeal may provide greater insight into the differences between these two closely related sites and the potential use of p16 in isolation. Future work may improve the understating of the role of HPV in pre-malignant and malignant oral lesions and may help to identify those lesions that are likely to progress to OSCC. These high risk patients could then be targeted for closer follow up and there may be a role for vaccination against HPV. However, vaccination is a contentious issue in that it would be difficult to identify at risk groups.

# REFERENCES


1. Acay R., Rezende N., Fontes A., Aburad A., Nunes F. and Sousa S. Human papillomavirus as a risk factor in oral carcinogenesis: a study using in situ hybridization with signal amplification. *Oral Microbiol Immun* 2008: **23**(4): 271-274

2. Adelstein D.J. and Rodriguez C.P. Human papillomavirus: Changing paradigms in oropharyngeal cancer. *Curr Oncol Rep* 2010; **12**(2): 115-120

3. Adewole R.A. Alcohol, smoking and oral cancer. A 10-year retrospective study at Base Hospital, Yaba. *West Afr J Med* 2002; **21**(2): 142-145

4. AJCC Cancer Staging Handbook, 2010. Seventh Ed. Springer-Verlag, New York, Inc.

5. Akanuma D., Uzawa N., Yoshida M.A., Negishi A., Amagasa T. and Ikeuchi, T. Inactivation patterns of the p16 (INK4a) gene in oral squamous cell carcinoma cell lines. *Oral Oncol* 1999; **35**(5): 476-483

6. Almadori G., Bussu F., Cadoni G., Galli J., Rigante M., Artuso A. and Maurizi M. Multistep laryngeal carcinogenesis helps our understanding of the field cancerisation phenomenon: a review. *Eur J Cancer* 2004; **40**(16): 2383-2388

7. Al-Swiahb J.N., Huang C.-C., Fang F.-M., Chuang H.-C., Huang H.-Y., Luo S.-D., Chen C.-H., Chen C.-M. and Chien C.-Y. Prognostic impact of p16, p53, epidermal growth factor receptor, and human papillomavirus in oropharyngeal cancer in a betel nut-chewing area. *Arch Otolaryngol* 2010; **136**(5): 502-508

8. Amaral T.M.P., da Silva Freire A.R., Carvalho A.L., Pinto C.A.L. and Kowalski L.P. Predictive factors of occult metastasis and prognosis of clinical stages I and II squamous cell carcinoma of the tongue and floor of the mouth. *Oral Oncol* 2004; **40**(8): 780-786

9. Androphy E.J. Molecular biology of human papillomavirus infection and oncogenesis. *J Invest Dermatol* 1994; **103**(2): 248-256

10. Angiero F., Berenzi A., Benetti A., Rossi E., Sordo R.D., Sidoni A., Stefani M. and Dessy E. Expression of P16, P53 and Ki-67 proteins in the progression of epithelia dysplasia of the oral cavity. *Anticancer Res* 2008; **28**(5A): 2535-2540

11. Angiero F., Farronato G., Dessy E., Magistro S., Seramondi R., Farronato D., Benedicenti S. and Tetè S. Evaluation of the cytotoxic and genotoxic effects of the orthodonthic bonding adhesives upon human gingival papillae through immunohistochemical expression of p53, p63 and p16. *Anticancer Res* 2009; **29**(10): 3983-3988

12. Arduino P.G., Surace A., Carbone M., Elia A., Massolini G., Gandolfo S. and Broccoletti R. Outcome of oral dysplasia: a retrospective hospital-based study of 207 patients with a long follow-up. *J Oral Pathol Med* 2009; **38**(6): 540-544

13. Argiris A., Karamouzis M., Raben D. and Ferris R.L. Head and neck cancer-*Seminar Lancet* 2008; **371**(9625): 1695-1709

14. Aulmann S., Schleibaum J., Penzel R., Schirmacher P., Gebauer G. and Sinn H.P. Gains of chromosome region 3q26 in intraepithelial neoplasia and invasive squamous cell carcinoma of the vulva are frequent and independent of HPV status. *J Clin Pathol* 2008; **61**(9):1034-1037

15. Bagán J.V., Murillo J., Poveda R., Gavaldá C., Jiménez Y. and Scully C. Proliferative verrucous leukoplakia: unusual locations of oral squamous cell carcinomas, and field cancerization as shown by the appearance of multiple OSCCs. *Oral Oncol* 2004; **40**(4): 440-443

16. Bagán J.V. and Scully C. Recent advances in oral oncology 2008; squamous cell carcinoma aetiopathogenesis and experimental studies. *Oral Oncol* 2009; **45**(7): e45-e48

17. Baker T.S., Newcomb W.W., Olson N.H., Cowsert L.M., Olson C. and Brown J.C. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys J* 1991; **60**(6): 1445-1456

18. Bailey J.A. *Concise dictionary of medical-legal terms*. New York; The Parthanon Publishing Group, 1998.

19. Bànkfalvi A. and Piffkò J. Prognostic and predictive factors in oral cancer: the role of the invasive tumour front. *J Oral Pathol Med* 2000; **29**(7): 291-298

20. Baleriola C., Millar D., Melki J., Coulston N., Altman P., Rismanto N. and Rawlinson W. Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections. *J Clin Virol* 2008; **42**(1): 22-26

21. Batsakis J.G. 2003. 3. Clinical pathology of oral cancer, In: Oral cancer. 1st Ed. Shah J. P. Johnson N.W. and Batsakis J.G., pp. 75-128. Martin Dunitz, Taylor Francis Group, UK.

22. Bauer H.M., Ting Y., Greer C.E., Chambers J.C., Tashiro C.J., Chimera J., Reingold A. and Manos M.M. Genital human papillomavirus infection in female university students as determined by a PCR-based method. *JAMA* 1991; **265**(4): 472-477

23. Bedi G., Westra W., Gabrielson E., Koch W. and Sidransky D. Multiple head and neck tumors: evidence for a common clonal origin. *Cancer Res* 1996; **56**(11): 2484-2487

24. Begum S., Cao D., Gillison M., Zahurak M., Westra W.H. Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clin Cancer Res* 2005; **11**(16): 5694-5699

25. Bellon M. and Nicot C. Regulation of telomerase and telomeres: Human tumor viruses take control. *J Natl Cancer I* 2008; **100**(2): 98-108

26. Benner S.E., Pajak T.F., Lippmann S.M., Early C. and Hong W.K. Prevention of second primary tumors with isotretinoin in patients with squamous cell carcinoma of the head and neck: long-term follow-up. *J Natl Cancer I* 1994; **86**(2): 140-141

27. Bettendorf O., Bànkfalvi A. and Piffkò J. Prognostic and predictive factors in oral squamous cell cancer: important tools for planning individual therapy? *Oral Oncol* 2004; **40**(2): 110-119

28. Bhonsle R.B., Pindborg J.J., Gupta P.C., Murti P.R. and Mehta Fali S. Incidence rate of oral lichen planus among Indian villagers. *Acta Dermato-Venereologica* 1979; **59**(3): 255-257

29. Bilde A., von Buchwald C., Dabelsteen E., Therkildsen M.H. and Dabelsteen S. Molecular markers in the surgical margin of oral carcinomas. *J Oral Pathol Med* 2009; **38**(1): 72-78

30. Binahmed A., Nason R.W. and Abdoh A.A. The clinical significance of the positive surgical margin in oral cancer. *Oral Oncol* 2007; **43**(8): 780-784

31. Bloching M., Hofmann A., Berghaus A., Lautenschläger C. and Grummt T. Mikrokerne als Biomarker zum Nachweis der Feldkanzerisierung im oberen Aerodigestivtrakt (Field cancerisation in the upper aerodigestive tract detected with the micronucleus assay). *HNO* 2000; **48**(6): 444-450

32. Boffetta P., Hecht S., Gray N., Gupta P. and Straif K. Smokeless tobacco and cancer. *Lancet Oncol* 2008; **9**(7): 667-75

33. Bohn O.L., Navarro L., Saldivar J. and Sanchez-Sosa S. Identification of human papillomavirus in esophageal squamous papillomas. *World J Gastroenterol* 2008; **14**(46): 7107-7111

34. Bouda M., Gorgoulis V.G., Kastrinakis N.G., Giannoudis A., Tsoli E., Danassi-Afentaki D., Foukas P., Kyroudi A., Laskaris G., Herrington C.S. and Kittas C. 'High risk' HPV types are frequently detected in potentially malignant and malignant oral lesions, but not in normal oral mucosa. *Modern Pathol* 2000; **13**(6): 644-653

35. Braakhuis B.J.M., Tabor M.P., Leemans C.R., van der Waal I., Snow G.B. and Brakenhoff R.H. Second primary tumors and field cancerization in oral and oropharyngeal cancer: Molecular techniques provide new insights and definitions. *Head Neck* 2002; **24**(2): 198-206

36. Braakhuis B.J.M., Tabor M.P., Kummer J.A., Leemans C.R. and Brakenhoff R.H. A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* 2003; **63**(8): 1727-1730

37. Braakhuis B.J.M., Leemans C.R. and Brakenhoff R.H. Expanding fields of genetically altered cells in head and neck squamous carcinogenesis. *Semin Cancer Biol* 2005; **15**(2): 113-120

38. Bradley K.T., Budnick S.D. and Logani S. Immunohistochemical detection of $p16^{INK4a}$ in dysplastic lesions of the oral cavity. *Modern Pathol* 2006; **19**(10): 1310-1316

39. Brandao V.C.R.A.B., Lacerda H.R., Lucena-Silva N. and Ximenes R.A.A. Frequency and types of human papillomavirus among pregnant and non-pregnant women with human immunodeficiency virus infection in Recife determined by genotyping. *Mem I Oswaldo Cruz* 2009; **104**(5): 755-763

40. Brandwein-Gensler M., Teixeira M.S., Lewis C.M., Lee B., Rolnitzky L., Hille J.J., Genden E., Urken M.L. and Wang B.Y. Oral squamous cell carcinoma: histologic risk assessment, but not margin status, is strongly predictive of local disease-free and overall survival. *Am J Surg Pathol* 2005; **29**(2): 167-78

41. Braganca J.F.¸ Sarian L.O., Pitta D.R., Maito A.B., Vassallo J., Pignataro F., Andrade L.A. and Derchain S. Expression of $p16^{INK4a}$ and cervical infection with high-risk human papillomavirus are not related to p53 activity in cervical intraepithelial neoplasia. *Int J Gynecol Cancer* 2008; **18**(5): 1060-1064

42. Brebi M.P., Ili G.C.G., López M.J., García M.P., Melo A.A., Montenegro H.S., Leal R.P., Guzmán G.P. and Roa S.J.C. Detection and genotyping of human papillomavirus in biopsies of uterine cervical adenocarcinoma *Rev Med Chile* 2009; **137**(3): 377-382

43. Breslow A. Thickness, cross-sectional area and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg* 1970; **172**(5): 902-908

44. Brieger J., Jacob R., Riazimand H.S., Essig E., Heinrich U.R., Bittinger F. and Mann W.J. Chromosomal aberrations in premalignant and malignant squamous epithelium. *Cancer Genet Cytogen* 2003; **144**(2): 148-155

45. Brismar S., Johansson B., Borjesson M., Arbyn M. and Andersson S. Follow-up after treatment of cervical intraepithelial neoplasia by human papillomavirus genotyping *Am J Obstet Gynecol* 2009; **201**(1): 17.e1-17.e8

46. Buajeeb W., Poonsawat S., Punyasingh J. and Sanguansin S. Expression of p16 in oral cancer and premalignant lesions. *J Oral Pathol Med* 2009; **38**(1): 104-108

47. Bundgaard T., Wildt J. and Elbrond O. Oral squamous cell cancer in non-users of tobacco and alcohol. *Clin Otolaryng Allied Sci* 1994; **19**(4): 320-326

48. Bundgaard T., Bentzen S.M. and Wildt J.W. Consumption of tobacco and alcohol as prognostic factors in oral cancer [Tobaks- og alkoholforbrug som prognostiske faktorer ved oral cancer.]. *Ugeskrift for Laeger* 1995; **157**(35): 4819-4822

49. Califano J., van der Riet P., Westra W., Nawroz H., Clayman G., Piantadosi S., Corio R., Lee D., Greenberg B., Koch W. and Sidransky D. Genetic progression model for head and neck cancer: Implications for field cancerization. *Cancer Res* 1996; **56**(11): 2488-2492

50. Califano J., Leong P.J., Koch W.M., Eisenberger C.F., Sidransky D. and Westra W.H. Second esophageal tumors in patients with head and neck squamous cell carcinoma: An assessment of clonal relationships. *Clin Cancer Res* 1999; **5**(7): 1862-1867

51. Califano J., Koch W., Sidransky D. and Westra W.H. Inverted sinonasal papilloma: A molecular genetic appraisal of its putative status as a precursor to squamous cell carcinoma. *Am J Pathol* 2000; **156**(1): 333-337

52. Campisi G., Panzarella V., Giuliani M., Lajolo C., Di Fede O., Falaschini S., Di Liberto C., Scully C. and Lo Muzio, L. Human papillomavirus: Its identikit and controversial role in oral oncogenesis, premalignant and malignant lesions. *Int J Oncol* 2007; **30**(4): 813-823

53. Campos E.A., Simões J.A., Rabelo-Santos S.H., Sarian L.O., Pitta D.R., Levi J.E. and Derchain S. Recovery of DNA for the detection and genotyping of human papillomavirus from clinical cervical specimens stored for up to 2 years in a universal collection medium with denaturing reagent. *J Virol Methods* 2008; **147**(2): 333-337

54. Cancela M.D.C., Ramadas K., Fayette J.-M., Thomas G., Muwonge R., Chapuis F., Thara S., Sankaranarayanan R. and Sauvaget C. Alcohol intake and oral cavity cancer risk among men in a prospective study in Kerala, India. *Community Dent Oral* 2009; **37**(4): 342-349

55. Cann A.J. *Principles of molecular virology*. 2$^{nd}$ ed. Academic Press, San Diego, 1997; pp. 1-210

56. Canova C., Hashibe M., Simonato L., Nelis M., Metspalu A., Lagiou P., Trichopoulos D., Ahrens W., igeot I., Merletti F., Richiardi L., Talamini R., Barzan L., Macfarlane G.J., Macfarlane T.V., Holcátová I., Bencko V., Benhamou S., Bouchardy C., Kjaerheim K., Lowry R., Agudo A., Castellsague´ X., Conway D.I., McKinney P.A., Znaor A., McCartan B.E., Healy C.M., Marron M. and Brennan P. Genetic Associations of 115 Polymorphisms with Cancers of the Upper Aerodigestive Tract across 10 European Countries: The ARCAGE Project. *Cancer Res* 2009; **69**(7): 2956-2965

57. Canto M.T., Goodman H.S., Horowitz A.M., Watson M.R. and Durán-Medina C. Latino Youths' Knowledge of Oral Cancer and Use of Tobacco and Alcohol. *Am J Health Behav* 1998; **22**(6): 411-420

58. Cao D.F., Begum S., Ali S.Z. and Westra W.H. Expression of p16 in benign and malignant cystic squamous lesions of the neck. *Hum Pathol* 2010; **41**(4): 535-539

59. Cao J., Zhang Z.Y., Patima Zhang Y.X. and Chen W.T. Human papillomavirus infection and p53 alteration in oral squamous cell carcinoma. *The Chinese Journal of Dental Research : The Official Journal of The Scientific Section of the Chinese Stomatological Association (CSA)* 2000; **3**(3): 44-49

60. Cao J., Zhou J., Gao Y., Gu L., Meng H., Liu H. and Deng D. Methylation of p16 CpG Island Associated with Malignant Progression of Oral Epithelial Dysplasia: A Prospective Cohort Study. *Clin Cancer Res* 2009; **15**(16): 5178-83

61. Carter R.L., Barr L.C., O'Brien C.J., Soo K.C. and Shaw H.J. Transcapsular spread of metastatic squamous cell carcinoma from cervical lymph nodes. *Am J Surg* 1985; **150**(4): 495-499

62. Casabonne D., Michael K.M., Waterboer T., Pawlita M., Forlund O., Burk R.D., Travis R.C., Key T.J. and Newton R. A prospective pilot study of antibodies against human papillomaviruses and cutaneous squamous cell carcinoma nested in the Oxford component of the European Prospective Investigation into Cancer and Nutrition. *Int J Cancer* 2007; **121**(8): 1862-1868

63. Castle P.E., Porras C., Quint W.G., Rodriguez A.C., Schiffman M., Gravitt P.E., González P., Katki H.A., Silva S., Freer E., Van Doorn L., Jiménez S., Herrero R. and Hildesheim A. Comparison of Two PCR-Based Human Papillomavirus Genotyping Methods. *J Clin Microbiol* 2008; **46**(10): 3437-3445

64. Castle P.E., Sadorra M., Lau T., Aldrich C., Garcia F.A.R. and Kornegay J. Evaluation of a Prototype Real-Time PCR Assay for Carcinogenic Human Papillomavirus (HPV) Detection and Simultaneous HPV Genotype 16 (HPV16) and HPV18 Genotyping. *J Clin Microbiol* 2009; **47**(10): 3344-3347

65. Castellsagué X., Munoz N., De Stefani E., Victora C.G., Castelletto R., Rolón P.A. and Jesús Quintana M. Independent and joint effects of tobacco smoking and alcohol drinking on the risk of esophageal cancer in men and women. *Int J Cancer* 1999; **82**(5): 657-664

66. Chacón J., Sanz I., Rubio M.D., De La Morena M.L., Díaz E., Mateos M.L. and Baquero F. Detection and genotyping of high-risk human papillomavirus in cervical specimens. *Enfermedades Infecciosas y Microbiologia Clinica* 2007; **25**(5): 311-316

67. Chang J.Y.-F., Lin M.-C. and Chiang C.-P. High-Risk Human Papillomaviruses May Have an Important Role in Non-Oral Habits-Associated Oral Squamous Cell Carcinomas in Taiwan. *Am J Clin Pathol* 2003; **120**(6): 909-916

68. Charfi L., Jouffroy T., de Cremoux P., Peltier N.L., Thioux M., Fréneaux P., Point D., Girod A., Rodriguez J. and Sastre-Garau X. Two types of squamous cell carcinoma of the palatine tonsil characterized by distinct etiology, molecular features and outcome. *Cancer Lett* 2008; **260**(1-2): 72-78

69. Chaudhary A.J., Singh M., Sundaram S. and Mehrotra R. Role of human papillomavirus and its detection in potentially malignant and malignant head and neck lesions: updated review. *Head Neck Oncol* 2009; **1**: 22

70. Chen C., Ricks S., Doody D.R., Fitzgibbons E.D., Porter P.L. and Schwartz S.M. N-acetyltransferase 2 polymorphisms, cigarette smoking and alcohol consumption, and oral squamous cell cancer risk. *Carcinogenesis* 2001; **22**(12): 1993-1999

71. Chen Y.W., Yu E.H., Wu T.H., Lo W.L., Li W.Y. and Kao S.Y. Histopathological factors affecting nodal metastasis in tongue cancer: analysis of 94 patients in Taiwan. *Int J Oral Max Surg* 2008; **37**(10): 912-916

72. Choi S.Y. and Kahyo H. Effect of cigarette smoking and alcohol consumption in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Epidemiol* 1991; **20**(4): 878-885

73. Chow L.T., Broker T.R. and Steinberg B.M. The natural history of human papillomavirus infections of the mucosal epithelia. *APMIS* 2010; **118**(6-7): 422-449

74. Chuang S.-C., Scelo G., Tonita J.M., Tamaro S., Jonasson J.G., Kliewer E.V., Hemminki K., Weiderpass E., Pukkala E., Tracey E., Friis S., Pompe-Kirn V., Brewster D.H., Martos C., Chia K.-S., Boffetta P., Brennan P. and Hashibe M. Risk of second primary cancer among patients with head and neck cancers: A pooled analysis of 13 cancer registries. *Int J Cancer* 2008; **123**(10): 2390-2396

75. Ciccolini F. Di Pasquale G., Carlotti F., Crawford L. and Tommasino M. Functional studies of E7 proteins from different HPV types. *Oncogene* 1994; **9**(9): 2633-2638

76. Ciechanover A. The ubiquitin-mediated proteolytic pathway: mechanisms of action and cellular physiology. *Biol Chem Hoppe-Seyler* 1994; **375**(9): 565-581

77. Cloos J., Leemans C.R., van der Sterre M.L.T., Kuik D.J., Snow G.B. and Braakhuis B.J.M. Mutagen sensitivity as a biomarker for second primary tumors after head and neck squamous cell carcinoma. *Cancer Epidem Biomar* 2000; **9**(7): 713-717

78. Coakes S.J. and Steed L.G. SPSS: Analysis without Anguish, version 10.0 for Windows, 2001; John Wiley & Sons, Australia Ltd.

79. Cody II D.T., Huang Y.H., Darby C.J., Johnson G.K. and Domann F.E. Differential DNA methylation of the p16$^{INK4A}$/CDKN2A promoter in human oral cancer cells and normal human oral keratinocytes. *Oral Oncol* 1999; **35**(5): 516-522

80. Coombs N.J., Gough A.C. and Primrose J.N. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res* 1999; **27**(16): e12

81. Copper M.P., Braakhuis B.J.M., De Vries N., Van Dongen G.A.M.S., Nauta J.J.P. and Snow G.B. A Panel of biomarkers of carcinogenesis of the upper aerodigestive tract as potential intermediate endpoints in chemoprevention trials. *Cancer* 1993; **71**(3): 825-830

82. Cortese A., Savastano G., Buccico R., Gargiulo M., Sica G. and Marracino M. Postoperative treatment of alcohol withdrawal syndrome in a patient operated for oral cancer [Terapia della sindrome da astinenza da alcol nel postoperatorio in paziente trattato chirurgicamente per carcinoma orale.]. *Minerva stomatologica* 2000; **49**(6): 327-331

83. Costa A.D.L.L, de Araújo Júnior R.F. and Ramos C.C.F. Correlation between TNM classification and malignancy histological feature of oral squamous cell carcinoma. *Rev Bras Otorrinolaringol* 2005; **71**(2): 181-187

84. Cubie H.A., Seagar A.L., McGoogan E., Whitehead J., Brass A., Arends M.J. and Whitley M.W. Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18. *J Clin Mol Pathol* 2001; **54**(1): 24-29

85. Culp T.D. and Christensen N.D. Kinetics of in vitro adsorption and entry of papillomavirus virions. *Virology* 2004; **319**(1): 152-61

86. Cunningham L.L., Pagano G.M., Li M., Tandon R., Holm S.W., White D.K. and Lele S.M. Overexpression of $p16^{INK4}$ is a reliable marker of human papillomavirus–induced oral high-grade squamous dysplasia. *Oral Surg Oral Med O* 2006; **102**(1): 77-81

87. Cuschieri K.S., Cubie H.A., Whitley M.W., Gilkison G., Arends M.J., Graham C. and McGoogan E. Persistent high risk HPV infection associated with development of cervical neoplasia in a prospective population study. *J Clin Pathol* 2005; **58**(9): 946-950

88. Dahlstrom K.R., Little J.A., Zafereo M.E., Lung M., Wei Q. and Sturgis E.M. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: A descriptive epidemiologic study. *Head Neck* 2008; **30**(1): 75-84

89. Dahlstrom K.R., Li G., Tortolero-Luna, G., Wei Q. and Sturgis E.M. Differences in history of sexual behaviour between patients with oropharyngeal squamous cell carcinoma and patients with squamous cell carcinoma at other head and neck sites. *Head Neck* 2011; **33**(6): 847-855

90. Dalstein V., Merlin S., Bali C., Saunier M., Dachezd R., Ronsin C. Analytical evaluation of the PapilloCheck test, a new commercial DNA chip for detection and genotyping of human papillomavirus. *J Virol Methods* 2009; **156**(1-2): 77-83

91. Das S.K., Nayak P. and Vasudevan D.M. Biochemical markers for alcohol consumption. *Indian Journal of Clinical Biochemistry* 2003; **18**(2): 111-118

92. Davidson B.J. 1. Epidemiology and Etiology, in Shah J.P., Patel S.G. and Atlas of Clinical Oncology: *Cancer of the Head and Neck*, ACS BC Decker Inc. 2001 pp. 1-18

93. Davies R., Hicks R., Crook T., Morris J. and Vousden K. Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* 1993; **67**(5): 2521-2528

94. Day G.L., Blot W.J., Austin D.F., Bernstein L., Greenberg R.S., Preston-Martin S., Schoenberg J.B., Winn D.M., McLaughlin J.K. and Fraumeni Jr. J.F. Racial differences in risk of oral and pharyngeal cancer: Alcohol, tobacco, and other determinants. *J Natl Cancer I* 1993; **85**(6): 465-473

95. Day G.L., Blot W.J., Shore R.E., McLaughlin J.K., Austin D.F., Greenberg R.S., Liff J.M., Preston-Martin S., Sarkar S., Schoenberg J.B. and Fraumeni Jr. J.F. Second cancers following oral and pharyngeal cancers: Role of tobacco and alcohol. *J Natl Cancer I* 1994; **86**(2): 131-137

96. Dayyani F., Etzel C.J., Liu M., Ho C-H., Lippman S.M. and Tsao A.S. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). *Head Neck Oncol* 2010; **2**(1): 15

97. de Antonio J.C., Fern´andez-Olmos A., Mercadillo M., Lindemann M.L.M. and Mochales F.B. Detection of high-risk human papillomavirus by two molecular techniques: Hybrid capture and linear array. *J Virol Methods* 2008; **149**(1): 163-166

98. de Boer M.F., Sanderson R.J., Damhuis R.A.M., Meeuwis C.A. and Knegt P.P. The effects of alcohol and smoking upon the age, anatomic sites and stage in the development of cancer of the oral cavity and oropharynx in females in the south west Netherlands. *Eur Arch Oto-Rhino-L* 1997; **254**(4): 177-179

99. de Koning M.N.C., Quint W.G.V., Struijk L., Kleter B., Wanningen P., van Doorn L., Weissenborn S.J., Feltkamp M. and ter Schegget J. Evaluation of a Novel Highly Sensitive, Broad-Spectrum PCR-Reverse Hybridization Assay for Detection and Identification of Beta-Papillomavirus DNA. *J Clin Microbiol* 2006; **44**(5): 1792-1800

100. de Koning M.N.C., Struijk L., Bavinck J.N.B., Kleter B., ter Schegget J. and Quint W.G.V. Betapapillomaviruses frequently persist in the skin of healthy individuals. *J Gen Virol* 2007; **88**(5): 1489-1495

101. de Koning M.N.C., Quint W.G.V and Pirog E.C. Prevalence of mucosal and cutaneous human papillomaviruses in different histologic subtypes of vulvar carcinoma. *Modern Pathol* 2008; **21**(3): 334-344

102. de Koning M.N.C., Waddell K., Magyezi J., Purdie K., Proby C., Harwood C., Lucas S., Downing R., Quint W.G.V. and Newton R. Genital and cutaneous human papillomavirus (HPV) types in relation to conjunctival squamous cell neoplasia: A case control study in Uganda. *Infectious Agents and Cancer* 2008; **3**(1): 12

103. de Roda Husman A., Walboomers J.M.M., van den Brule A.J.C., Meijer C.J.L.M. and Snidjers P.J.F. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR *J Gen Virol* 1995; **76**(4): 1057-1062

104. de Villiers E.M., Fauquet C., Broker T.R., Bernard H.U. and Zur Hausen H. Classification of papillomaviruses. *Virology* 2004; 324: 17-27

105. de Villiers E.M. and Gunst K. Characterization of seven novel human papillomavirus types isolated from cutaneous tissue, but also present in mucosal lesions. *J Gen Virol* 2009; 90: 1999-2004

106. Doeberitz M.K. New marker for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infection. *Eur J Cancer* 2002; **38**(17): 2220-2242

107. Doorbar J., Ely S., Sterling J., McLean C. and Crawford L. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* 1991; **352**(6338): 824-827

108. Doorbar J. The papillomavirus life cycle. *J Clin Virol* 2005; **32S**: S7-S15

109. D'Souza G., Kreimer A.R., Viscidi R., Pawlita M., Fakhry C., Koch W.M., Westra W.H. and Gillison M.L. Case–Control Study of Human Papillomavirus and Oropharyngeal Cancer. *N Engl J Med* 2007; **356**(19): 1944-1956

110. Duncan L., Jacob S. and Hubbard E. Evaluation of p16INK4a as a Diagnostic Tool in the Triage of Pap Smears Demonstrating Atypical Squamous Cells of Undetermined Significance. *Cancer* 2008; **114**(1): 34-48

111. Egawa K. Do human papillomaviruses target epidermal stem cells? *Dermatology* 2003; **207**(3): 251-254

112. Elwood J.M., Pearson J.C.G., Skippen D.H. and Jackson S.M. Alcohol, smoking, social and occupational factors in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Cancer* 1984; **34**(5): 603-612

113. Eversole L.R. Dysplasia of the Upper Aerodigestive Tract Squamous Epithelium. *Head and Neck Pathol* 2009; **3**(1): 63-68

114. Erali M., David C. Pattison D.C., Carl T. Wittwer C.T. and Petti C.A. Human Papillomavirus Genotyping Using an Automated Film-Based Chip Array. *Journal of Molecular Diagnostics* 2009; **11**(5): 439-445

115. Fabian M.C., Irish J.C., Brown D.H., Liu T.C. and Gullane P.J. Tobacco, alcohol, and oral cancer: The patient's perspective. *J Otolaryngol* 1996; **25**(2): 88-93

116. Fakhry C. and Gillison M.L. Clinical implications of human papillomavirus in head and neck cancers. *J Clin Oncol* 2006; **24**(17): 2602-2610

117. Farshadpour F., Hordijk G.J., Koole R. and Slootweg P.J. Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. *Oral Dis* 2007; **13**(2): 239-243

118. Feltkamp M.C.W., de Koning M.N.C., Bavinck J.N.B. and ter Schegget J. Betapapillomaviruses: Innocent bystanders or causes of skin cancer. *J Clin Virol* 2008; **43**(4): 353-360

119. Ferreira Santos A.L., Mauricette Derchain S.F., Martins M.R., Zanatta Sarian L.O., Zangiacome Martinez E. and Syrjänen K.J. Human papillomavirus viral load in predicting high-grade CIN in women with cervical smears showing only atypical squamous cells or low-grade squamous intraepithelial lesion. *Sao Paulo Med J* 2003; **121**(6): 238-243

120. Fey M.C. and Beal M.W. Role of human papilloma virus testing in cervical cancer prevention. *J Midwifery Women's Health* 2004; **49**(1): 4-13

121. Figaro Gattá G.J., De Carvalho M.B., Siraque M.S., Curioni O.A., Kohler P., Eluf-Neto J. and Wünsch-Filho V. Genetic polymorphisms of CYP1A1, CYP2E1, GSTM1, and GSTT1 associated with head and neck cancer. *Head Neck* 2006; **28**(9): 819-826

122. Figuero Ruiz E., Carretero Peláez M.Á., Cerero Lapiedra R., Esparza Gómez G. and Moreno López L.A. Effects of the consumption of alcohol in the oral cavity: Relationship with oral cancer [Efectos del consumo de alcohol etílico en la cavidad oral: Relación con el cáncer oral]. *Medicina Oral* 2004; **9**(1): 14-23

123. Fischer C.A., Zlobec I., Green E., Probst S., Storck C., Lugli A., Tornillo L., Wolfensberger M. and Terracciano L.M. Is the improved prognosis of p16 positive oropharyngeal squamous cell carcinoma dependent of the treatment modality. *Int J Cancer* 2010; **126**(5): 1256-1262

124. Flores-Munguia R., Siegel E., Klimecki W.T. and Giuliano A.R. Performance Assessment of Eight High-Throughput PCR Assays for Viral Load Quantitation of Oncogenic HPV Types. *Journal of Molecular Diagnostics* 2004; **6**(2): 115-124

125. Florin L., Sapp C., Streeck R.E. and Sapp M. Assembly and translocation of papillomavirus capsid proteins. *J Virol* 2002; **76**(19): 10009-100014

126. Fontaine J., Hankins C., Moneye D., Rachlis A., Pourreaux K., Ferenczyg A. and Coutlée F. Human papillomavirus type 16 (HPV-16) viral load and persistence of HPV-16 infection in women infected or at risk for HIV. *J Clin Virol* 2008; **43**(3): 307-312

127. Foulkes W.D., Flanders T.Y., Pollock P.M. and Hayward N.K. The CDKN2A (pl6) gene and human cancer. *Mol Med* 1997; **3**(1): 5-20

128. Franceschi S., Levi F., La Vecchia C., Conti E., Dal Maso L., Barzan L. and Talamini R. Comparison of the effect of smoking and alcohol drinking between oral and pharyngeal cancer. *Int J Cancer* 1999; **83**(1): 1-4

129. Franceschi S., Levi F., Dal Maso L., Talamini R., Conti E., Negri E. and La Vecchia, C. Cessation of alcohol drinking and risk of cancer of the oral cavity and pharynx. *Int J Cancer* 2000; **85**(6): 787-790

130. Fregonesi P.A.G., Teresa D.B., Duarte R.A., Neto C.B., de Oliveira M.R.B. and Soares C.P. $p16^{INK4A}$ immunohistochemical overexpression in premalignant and malignant oral lesions infected with human papillomavirus. *J Histochem Cytochem* 2003; **51**(10): 1291-1297

131. Friedrich R.E., Sperber C., Jäkel T., Röser K. and Löning T. Basaloid lesions of oral squamous epithelial cells and their association with HPV infection and p16 expression. *Anticancer Res* 2010; **30**(5): 1605-1612

132. Fujii T., Saito M. Iwata T., Hirao N., Nishio H., Ohno A., Tsukazaki K., Mukai M., Kameyama K. and Aoki D. Ancillary testing of liquid-based cytology specimens for identification of patients at high risk of cervical cancer. *Virchows Arch* 2008; **453**(6): 545-555

133. Fujita S., Senba M., Kumatori A., Hayashi T., Ikeda T. and Toriyama K. Human papillomavirus infection in oral verrucous carcinoma: Genotyping analysis and inverse correlation with p53 expression. *Pathobiology* 2008; **75**(4): 257-264

134. Fukui Y., Yamakawa T., Taniki T., Numoto S., Miki H. and Monden Y. Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. *Cancer* 2001; **92**(11): 2875-2884

135. Furniss C.S., McClean M.D., Smith J.F., Bryan J., Applebaum K.M., Nelson H.H., Posner M.R. and Kelsey K.T. Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use. *Ann Oncol* 2009; **20**(3): 534-541

136. Galan-Sanchez F. and Rodriguez-Iglesias M.A. Comparison of human papillomavirus genotyping using commercial assays based on PCR and reverse hybridization methods. *APMIS* 2009; **117**(10): 708-715

137. Galan-Sanchez F. and Rodriguez-Iglesias M.A. Use of cervista HPV HR assay for detection of human papillomavirus in samples with hybrid capture borderline negative results. *APMIS* 2010; **118**(9): 681-684

138. Galmiche L., Costle-Burel M., Lopes P, *et al*. The expression of p16INKa is not correlated with HPV status in CIN 1. *Histopathology* 2006; **48**(6): 767

139. Garcia S.B., Park H.S., Novelli M. and Wright N.A. Field cancerization, clonality, and epithelial stem cells: The spread of mutated clones in epithelial sheets. *J Pathol* 1999; **187**(1): 61-81

140. García-Sierra N., Martró E., Castellà E., Llatjós M., Tarrats A., Elisabet Bascuñana, Díaz R., Carrasco M., Sirera G., Matas L. and Vicente Ausina V. Evaluation of an Array-Based Method for Human Papillomavirus Detection and Genotyping in Comparison with Conventional Methods Used in Cervical Cancer Screening. *J Clin Microbiol* 2009; **47**(7): 2165-2169

141. Garnett T.O. and Duerksen-Hughes P.J. Modulation of apoptosis by human papillomavirus (HPV) oncoproteins. *Arch Virol* 2006; **151**(12): 2321-2335

142. Gillison M.L., D'Souza G., Westra W., Sugar E., Xiao W., Begum S. and Viscidi R. Distinct risk factor profiles for human papillomavirus type 16 – positive and human papillomavirus type 16 – negative head and neck cancers. *J Natl Cancer I* 2008; **100**(6): 407-420

143. Giordano G., Azzoni C., D'Adda T., Roccob A., Gnettia L., Froioa E., Merisioc C. and Melpignanoc M. Human papilloma virus (HPV) status, p16$^{INK4a}$, and p53 overexpression in epithelial malignant and borderline ovarian neoplasms. *Pathology – Research and Practice* 2008; **204**(3): 163-174

144. Giovannelli L., Campisi G., Colella G., Capra G., Di Liberto C., Caleca M.P., Matranga D., D'Angelo M., Lo Muzio L. and Ammatuna P. Brushing of oral mucosa for diagnosis of HPV infection in patients with potentially malignant and malignant oral lesions. *Molecular Diagnosis and Therapy* 2006; **10**(1): 49-55

145. Girja K.P., Sundharam B.S., Krishnan P.A. and Devi C.S. Biochemical changes of saliva in tobacco chewers tobacco smokers, alcohol consumers, leukoplakia and oral cancer patients. *Indian Journal of Dental Research* : official publication of Indian Society for Dental Research 2002; **13**(2): 102-107

146. Gonzalez J.V. , Gutierrez R.A., Keszler A., Colacino M. del C., Alonio L.V., Teyssie A.R. and Picconi M.A. Human papillomavirus in oral lesions. *Medicina* 2007; **67**(4): 363-368

147. González-Losa M.D.R., Manzano-Cabrera L., Rueda-Gordillo F., Hernández-Solís S.E. and Puerto-Solís, M. Low prevalence of high risk human papillomavirus in normal oral mucosa by hybrid capture 2. *Braz J Microbiol* 2008; **39**(1): 32-34

148. González-Moles M.A., Gil-Montoya J.A. and Ruiz-Ávila I. Bases moleculares de la cancerización de cavidad oral (Biomolecular of the field cancerization in oral cavity). *Avances En Odontoestomatologia* 2008; **24**(1): 55-60

149. Goon P.K.C., Stanley M.A., Ebmeyer J., Steinsträsser L., Upile T., Jerjes W., Manuel Bernal-Sprekelsen M., Görner M. and Sudhoff H.H. HPV & head and neck cancer: a descriptive update. *Head Neck Oncol* 2009; **1**: 36

150. Gospodarowicz M. and O'Sullivan B.: Prognostic Factors: Principles and Application, in Gospodarowicz M., Henson D.E. and Hutter R.V.P.: *Prognostic Factors in Cancer*. 2nd ed. New York; Wiley-Liss, 2001, pp. 17-36

151. Gospodarowicz M., O'Sullivan B, and Bristow: Host and Environment-related Prognostic Factors: Principles and Application, in Gospodarowicz M., Henson D.E. and Hutter R.V.P.: *Prognostic Factors in Cancer*. 2nd ed. New York; Wiley-Liss, 2001, pp. 71-94

152. Gospodarowicz M., O'Sullivan B and Koh E.S.: Host and Environment-related Prognostic Factors: Principles and Application, in Gospodarowicz M., O'Sullivan B. and Sobin L.H.: *Prognostic Factors in Cancer*. 3rd ed. New York; Wiley-Liss, 2006, pp. 23-34

153. Graham S., Dayal H. and Rohrer T. Dentition, diet, tobacco, and alcohol in the epidemiology of oral cancer. *J Natl Cancer I* 1977; **59**(6): 1611-1618

154. Greer Jr. R.O., Meyers A., Said S.M. and Shroyer K.R. Is $p16^{INK4a}$ protein expression in oral ST lesions a reliable precancerous marker? *Int J Oral Max Surg* 2008; **37**(9): 840-846

155. Guerrero D., Guarch R., Ojer A., Casas J.M., Ropero S., Mancha A., Pesce C., Lloveras B., Garcia-Bragado F. and Puras A. Hypermethylation of the thrombospondin-1 gene is associated with poor prognosis in penile squamous cell carcinoma. *BJU International* 2008; **102**(6): 747-755

156. Güneri P., Çankaya H., Yavuzer A., Güneri E.A., Erişen L., Özkul D., El Nehir S., Karakaya S., Arican A. and Boyacioğlu H. Primary oral cancer in a Turkish population sample: Association with sociodemographic features, smoking, alcohol, diet and dentition. *Oral Oncol* 2005; **41**(10): 1005-1012

157. Guo M., Sneige N., Silva E.G., Jan Y.J., Cogdell D.E., Lin E., Luthra R. and Zhang W. Distribution and viral load of eight oncogenic types of human papillomavirus (HPV) and HPV 16 integration status in cervical intraepithelial neoplasia and carcinoma. *Mod Pathol* 2007; **20**(2): 256-266

158. Guo X.L., Sun S.Z., Wang W.X., Wei F.C., Yu1 H.B. and Ma B.L. Alterations of $p16^{INK4a}$ tumour suppressor gene in mucoepidermoid carcinoma of the salivary glands. *Int J Oral Max Surg* 2007; **36**(4): 350-353

159. Gupta P.C., Mehta F.S. and Daftary D.K. Incidence rate of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. *Community Dent Oral Epidemiol* 1980; **8**(6): 287-333

160. Ha P.K. and Califano J.A. The molecular biology of mucosal field cancerization of the head and neck. *Crit Rev Oral Biol Med* 2003; **14**(5): 363-369

161. Hafkamp H.C., Speel E.J., Haesevoets A., Bot F.J., Dinjens W.N., Ramaekers F.C., Hopman A.H. and Manni J.J. A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of

p16INK4A and p53 in the absence of mutations in p53 exons 5-8. *Int J Cancer* 2003; **107**(3): 394-400

162. Hafkamp H.C., Manni J.J., Haesevoets A., Voogd A.C., Schepers M., Bot F.J., Hopman A.H.N., Ramaekers F.C.S. and Speel E.J.M. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. *Int J Cancer* 2008; **122**(12): 2656-2664

163. Hagensee M.E., Olson N.H., Baker T.S. and Galloway D.A. Three-dimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. *J Virol* 1994; **68**(7): 4503-4504

164. Haines D.M. and Chelack B.J. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. *J Vet Diagn Invest* 1991; **3**(1): 101-112

165. Hair J.F., Anderson R.E., Tatham R.L. and Black W.C. Multivariate Data Analysis 5th Edition, 1998; Prentice-Hall International, Inc.

166. Hall G.L., Shaw R.J., Field A., Rogers S.N., Sutton D.N., Woolgar J.A., Lowe D., Liloglou T., Field J.K. and Risk J.M. p16 Promoter Methylation Is a Potential Predictor of Malignant Transformation in Oral Epithelial Dysplasia. *Cancer Epidem Biomar* 2008; **17**(8): 2174-2179

167. Ham J., Dostatni N., Gauthier J.M. and Yaniv M. The papillomavirus E2 protein: a factor with many talents. *Trends Biochem Sci* 1991; **16**(11): 440-444

168. Haque R., Contreras R., McNicoll M.P., Eckberg E.C. and Petitti D.B. Surgical margins and survival after head and neck cancer surgery. *BMC Ear, Nose and Throat Disorders* 2006; **6**(2): 1-6

169. Harris E.L. Association of oral cancers with alcohol consumption: exploring mechanisms. *J Natl Cancer I* 1997; **89**(22): 1656-1657

170. Harty L.C., Caporaso N.E., Hayes R.B., Winn D.M., Bravo-Otero E., Blot W.J., Kleinman D.V., Brown L.M., Armenian H.K., Fraumeni Jr., J.F. and Shields P.G. Alcohol dehydrogenase 3 genotype and risk of oral cavity and pharyngeal cancers. *J Natl Cancer I* 1997; **89**(22): 1698-1705

171. Harwood C.A., Spink P.J., Surentheran T., Leigh I.M., de Villier E-M., McGregor J.M., Proby C.M. and Breuer J. Degenerate and nested PCR: a highly sensitive and specific method for detection of human papillomavirus infection in cutaneous warts. *J Clin Microbiol* 1999; **37**(11): 3545-3555

172. Hasegawa M., Nelson H.H., Peters E., Ringstrom E., Posner M. and Kelsey K.T. Patterns of gene promoter methylation in squamous cell cancer of the head and neck. *Oncogene* 2002; **21**(27): 4231-4236

173. Hashi A., Xu J.-Y., Kondo T., Hashi K., Yuminamochi T., Nara M., Murata S.-I., Katoh R. and Hoshi K. p16INK4a overexpression independent of human

papillomavirus infection in lobular endocervical glandular hyperplasia. *Int J Gynecol Pathol* 2006; **25**(2): 187-194

174. Hashibe M., Brennan P., Benhamou S., Castellsague X., Chen C., Curado M.P., Maso L.D., Daudt A.W., Fabianova E., Wünsch-Filho V., Franceschi S., Hayes R.B., Herrero R., Koifman S., La Vecchia C., Lazarus P., Levi F., Mates D., Matos E., Menezes A., Muscat J., Eluf-Neto J., Olshan A.F., Rudnai P., Schwartz S.M., Smith E., Sturgis E.M., Szeszenia-Dabrowska N., Talamini R., Wei Q., Winn D.M., Zaridze D., Zatonski W., Zhang Z.-F., Berthiller J. and Boffetta P. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *J Natl Cancer I* 2007; **99**(10): 777-789

175. Hayes R.B., Bravo-Otero E., Kleinman D.V., Brown L.M., Fraumeni Jr. J.F., Harty L.C. and Winn D.M. Tobacco and alcohol use and oral cancer in Puerto Rico. *Cancer Cause Control* 1999; **10**(1): 27-33

176. Hays G.L., Lippman S.M., Flaitz C.M., Brown R.S., Pang A., Devoll R. and Hong W.K. Co-carcinogenesis and field cancerization: oral lesions offer first signs. *J Am Dent Assoc* 1995; **126**(1): 47-51

177. He G.-R., Liu Q.-Q., Gong W.-B., Tang S.-M., Fang H.-H. and Liu A.-Q. Genotyping and quantization of human papillomavirus by using the multi-fluorescent quantitative PCR assay Chinese *J Microbiol Immunol* 2007; **27**(8): 762-765

178. Hebner C.M. and Laimins L.A. Human papillomavirus: Basic mechanisms of pathogenesis and oncogenicity. *Rev Med Virol* 2006; **16**(2): 83-97

179. Heinzel P.A., Balaram P. and Bernard H.U. Mutations and polymorphisms in the p53, p21 and p16 genes in oral carcinomas of Indian betel quid chewers. *Int J Cancer* 1996; **68**(4): 420-423

180. Herschfus L. The synergistic effect of alcohol and tobacco abuse on oral cancer. *The Journal of the Michigan Dental Association* 1991; **73**(2): 18-19

181. Hesselink A.T., van den Brule A.J.C., Groothuismink Z.M.A., Molano M.J. Berkhof J., Meijer C.J.L.M. and Snijders P.J.F. Comparison of Three Different PCR Methods for Quantifying Human Papillomavirus Type 16 DNA in Cervical Scrape Specimens. *J Clin Microbiol* 2005; **43**(9): 4868-4871

182. Hindle, I., Downer M.C., Moles D.R. and Speight P.M. Is alcohol responsible for more intra-oral cancer? *Oral Oncol* 2000; **36**(4): 328-333

183. Ho T., Wei Q. and Sturgis E.M. Epidemiology of carcinogen metabolism genes and risk of squamous cell carcinoma of the head and neck. *Head Neck* 2007; **29**(7): 682-99

184. Ho P.-S., Yang Y.-H., Shieh T.-Y., Huang I.-Y., Chen Y.-K., Lin K.-N. and Chen, C.-H. Consumption of areca quid, cigarettes, and alcohol related to the comorbidity of oral submucous fibrosis and oral cancer. *Oral Surg Oral Med O* 2007; **104**(5): 647-652

185. Hobbs C.G.L., Sterne J.A.C., Bailey M., Heyderman R.S., Birchall M.A. and Thomas S.J. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. *Clin Otolaryng* 2006; **31**(4): 259-266

186. Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Curr Opin Cell Biol* 1995; **7**(2): 215-223

187. Hoevenaars B.M., van der Avoort I.A.M., de Wilde P.C.M., Massuger L.F.A.G., Melchers W.J.G., de Hullu J.A. and Bulten J. A panel of $p16^{INK4A}$, MIB1 and p53 proteins can distinguish between the 2 pathways leading to vulvar squamous cell carcinoma. *Int J Cancer* 2008; **123**(12): 2767-2773

188. Holmes-McNary M. Impact factors on development of cirrhosis and subsequent hepatocellular carcinoma. *Compendium of continuing education in dentistry* (Jamesburg, N.J. : 1995) 2001; **22**(3): 19-33

189. Hong J.H., Lee J.K., Song E., Lee J. and Kim M.K. Evaluation of the compatibility of the Digene media when performing the Roche linear array human papillomavirus genotyping test. *Arch Gynecol Obstet* 2009; **280**(4): 613-618

190. Hooper S.J., Wilson M.J. and Crean S.J. Exploring the link between microorganisms and oral cancer: A systematic review of the literature. *Head Neck* 2009; **31**(9): 1228-1239

191. Hormia M., Wilberg J., Ruokonen H. and Syrjänen S. Marginal periodontium as a potential reservoir of human pappilomavirus in oral mucosa. *J Periodontol* 2005; **76**(3): 358-363

192. Horvath V.S.J., Boulet G.S.V., Renoux V.M., Delvenne P.O. and Bogers J-P. J. Mechanisms of cell entry by human papillomaviruses: an overview. *Virol J* 2010; **7**:11

193. Huang W.-Y., Winn D.M., Brown L.M., Gridley G., Bravo-Otero E., Diehl S.R., Fraumeni Jr., J.F. and Hayes R.B. Alcohol concentration and risk of oral cancer in Puerto Rico. *Am J Epidemiol* 2003; **157**(10): 881-887

194. Huang X., Pateromichelakis S., Hills A., Sherriff M., Lyons A., Langdon J., Odell E., Morgan P., Harrison J. and Partridge M. p53 Mutations in Deep Tissues Are More Strongly Associated with Recurrence than Mutation-Positive Mucosal Margins. *Clin Cancer Res* 2007; **13**(20): 6099-6106

195. Ide R., Mizoue T., Fujino Y., Hoshiyama Y., Sakata K., Tamakoshi A. and Yoshimura T. Cigarette smoking, alcohol drinking, and oral and pharyngeal cancer mortality in Japan. *Oral Dis* 2008; **14**(4): 314-319

196. Iftner T., Germ L., Swoyer R., Kjaer S.K., Breugelmans J.G., Munk C., Stubenrauch F., Antonello J., Bryan J.T. and Taddeo F.J. Study Comparing Human Papillomavirus (HPV) Real-Time Multiplex PCR and Hybrid Capture II INNO-LiPA v2 HPV Genotyping PCR Assays *J Clin Microbiol* 2009; **47**(7): 2106-2113

197. International Agency for Research against Cancer (IARC) Monographs on the Evaluation of Carcinogenic Risks to Humans. Tobacco Habits Other than Smoking; Betel-Quid and Areca-Nut Chewing; and Some Related Nitrosamines. IARC Lyon France, 1985; 37

198. International Agency for Research against Cancer (IARC) Monographs on the Evaluation of Carcinogenic Risks to Humans. Tobacco Smoking. IARC Lyon France, 1986; 38

199. International Agency for Research against Cancer (IARC) Monographs on the Evaluation of Carcinogenic Risks to Humans. Alcohol Drinking. IARC Lyon France, 1988; 44

200. International Agency for Research against Cancer. Human papillomaviruses. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 1995; 64

201. International Agency for Research against Cancer. Tobacco smoke and Involuntary Smoking. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 2004; 83

202. International Agency for Research against Cancer. Betel-quid and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 2004; 85

203. International Agency for Research against Cancer. Smokeless Tobacco. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 2006; 89

204. International Agency for Research against Cancer. Smokeless Tobacco and Some Tobacco-specific N-Nitrosamines. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 2007; 89

205. International Agency for Research against Cancer. Human papillomaviruses. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC Lyon France, 2007; 90

206. Ishida E., Nakamura M., Ikuta M., Shimada K., Matsuyoshi S., Kirita T. and Konishi N. Promotor hypermethylation of $p14^{ARF}$ is a key alteration for progression of oral squamous cell carcinoma. *Oral Oncol* 2005; **41**(6): 614-622

207. Jamison J., Wilson R.T. and Carson J. The evaluation of human papillomavirus genotyping in cervical liquid-based cytology specimens; using the Roche Linear Array HPV genotyping assay. *Cytopathology* 2009; **20**(4): 242-248

208. Jang S.J., Chiba I., Hirai A., Hong W.K. and Mao L. Multiple oral squamous epithelial lesions: are they genetically related? *Oncogene* 2001; **20**(18): 2235-2242

209. Jeronimo J., Wentzensen N., Long R., Schiffman M., Dunn S.T., Allen R.A., Walker J.L., Gold M.A., Zuna R.E., Sherman M.E., Wacholder S. and Wang S.S. Evaluation of linear array human papillomavirus genotyping using automatic optical imaging software. *J Clin Microbiol* 2008; **46**(8): 2759-2765

210. Jimenez C., Correnti M., Salma N., Cavazza M.E. and Perrone, M. Detection of human papillomavirus DNA in benign oral squamous epithelial lesions in Venezuela. *J Oral Pathol Med* 2001; **30**(7): 385-388

211. Johnson N.W. 1. Global Epidemiology, in Shah J.P., Johnson N.W. and Batsakis J.G. *Oral Cancer*, Martin Dunitz, Taylor & Francis Group. 2003 pp. 1-32

212. Johnston W.D. and Ballantyne A.J. Prognostic effect of tobacco and alcohol use in patients with oral tongue cancer. *Am J Surg* 1977; **134**(4): 444-447

213. Jones H.B., Sykes A., Bayman N., Sloan P., Swindell R., Patel M. and Musgrove B. The impact of lymphovascular invasion on survival in oral carcinoma. *Oral Oncol* 2009; **45**(1): 10-15

214. Kabat G.C., Chang C.J. and Wynder E.L. The role of tobacco, alcohol use, and body mass index in oral and pharyngeal cancer. *Int J Epidemiol* 1994; **23**(6): 1137-1144

215. Kane S.V., Gupta M., Kakade A.C. and D'Cruz A. Depth of invasion is the most significant histological predictor of subclinical cervical lymph node metastasis in early squamous carcinomas of the oral cavity. *EJSO* 2006; **32**(7): 795-803

216. Kansky A.A., Poljak M., Seme K., Kocjan B.J., Gale N., Luzar B. and Golouh R. Human papillomavirus DNA in oral squamous cell carcinomas and normal oral mucosa. *Acta Virol* 2003; **47**(1): 11-16

217. Kansky A.A., Seme K., Maver P.J., Luzar B., Gale N. and Poljak M. Human papillomaviruses (HPV) in tissue specimens of oral squamous cell papillomas and normal oral mucosa. *Anticancer Res* 2006; **26**(4B): 3197-3201

218. Katta R. Lichen planus. *Am Fam Physician*. 2000; **61**(11): 3319-3328

219. Kato K., Hara A., Kuno T., Mori H., Yamashita T., Toida M. and Shibata T. Aberrant promoter hypermethylation of p16 and MGMT genes in oral squamous cell carcinomas and the surrounding normal mucosa. *J Cancer Res Clin Oncol* 2006; **132**(11): 735-743

220. Katoh T., Kaneko S., Kohshi K., Munaka M., Kitagawa K., Kunugita N., Ikemura K. and Kawamoto T. Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and oral cavity cancer. *Int J Cancer* 1999; **83**(5): 606-609

221. Kerawala C.J. Oral cancer, smoking and alcohol: The patients' perspective. *Brit J Oral Max Surg* 1999; **37**(5): 374-376

222. Keski-Säntti H., Atula T., Tikka J., Hollmén J., Mäkitie A.A. and Leivo I. Predictive value of histopathologic parameters in early squamous cell carcinoma of oral tongue *Oral Oncol* 2007; **43**(10): 1007-1013

223. Khan F., Robinson P.G., Warnakulasuriya K.A.A.S., Newton J.T., Gelbier S. and Gibbons D.E. Predictors of tobacco and alcohol consumption and their relevance to oral cancer control amongst people from minority ethnic communities in the South Thames health region, England. *J Oral Pathol Med* 2000; **29**(5): 214-219

224. Khan M.J., Castle P.E., Lorincz A.T., Wacholder S., Sherman M., Scott D.R., Rush B.B., Glass A.G. and Schiffman M. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer I* 2005; **97**(14): 1072-1079

225. Kim J. and Chae C. Optimized protocols for the detection of porcine circovirus 2 DNA from formalin-fixed paraffin-embedded tissues using nested polymerase chain reaction and comparison of nested PCR with in situ hybridization. *J Virol Methods* 2001; **92**(2): 105-111

226. Klaassen C.H.W., Prinsen C.F.M., de Valk H.A., Horrevorts A.M., Jeunink M.A.F. and Thunnissen F.B.J.M. DNA Microarray Format for Detection and Subtyping of Human Papillomavirus. *J Clin Microbiol* 2004; **42**(5): 2152-2160

227. Kleter B., van Doorn L., ter Schegget J., Schrauwen L., van Krimpen K., Burger M., ter Harmsel B. and Quint W. Novel Short-Fragment PCR Assay for Highly Sensitive Broad-Spectrum Detection of Anogenital Human Papillomaviruses. *Am J Pathol* 1998; **153**(6): 1731-1739

228. Kleter B., van Doorn L., Schrauwen L., Molijn A., Sastrowijoto S., ter Schegget J., Lindeman J., ter Harmsel B., Burger M. and Quint W. Development and Clinical Evaluation of a Highly Sensitive PCR-Reverse Hybridization Line Probe Assay for Detection and Identification of Anogenital Human Papillomavirus. *J Clin Microbiol* 1999; **37**(8): 2508-2517

229. Klingenberg B., Hafkamp H.C., Haesevoets A., Manni J.J., Slootweg P.J., Weissenborn S.J., Klussmann J.P. and Speel E.-J.M. $P16^{INK4A}$ overexpression is frequently detected in tumour-free tonsil tissue without association with HPV. *Histopathology* 2010; **56**(7): 957-967.

230. Klinge U. and Fiebeler A. Analysis of survival curve configuration is relevant for determining pathogenesis and causation. *Med Hypotheses* 2009; **72**(5): 510-517

231. Klussmann J.P., Gültekin E., Weissenborn S.J., Wieland U., Dries V., Dienes H.P., Eckel H.E., Pfister H.J. and Fuchs P.G. Expression of p16 protein identifies

a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol* 2003; **162**(3): 747-753

232. Klussmann J.P., Weissenborn S.J., Wieland U., Dries V., Eckel H.E., Pfister H.J. and Fuchs P.G. Human papillomavirus-positive tonsillar carcinomas: a different tumor entity? *Med Microbiol Immunol* 2003; **192**(3): 129-132

233. Klussmann J.P., Mooren J.J., Lehnen M., Claessen S.M.H., Stenner M., Huebbers C.U., Weissenborn S.J., Wedemeyer I. and Preuss S.F. Genetic Signatures of HPV-related and Unrelated Oropharyngeal Carcinoma and Their Prognostic Implications. *Clin Cancer Res* 2009; **15**(5): 1779-1786

234. Kok L.F., Lee M.Y., Tyan Y.S., Wu T.S., Cheng Y.W., Kung M.F., Wang P.H. and Han C.P. Comparing the scoring mechanisms of $p16^{INK4a}$ immunohistochemistry based on independent nucleic stains and independent cytoplasmic stains in distinguishing between endocervical and endometrial adenocarcinomas in a tissue microarray study. *Arch Gynecol Obstet* 2010; **281**(2): 293-300

235. Kong C.S., Narasimhan B., Cao H., Kwok S., Erickson J.P., Koong A., Pourmand N. and Le Q. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. *Int J Radiat Oncol* 2009; **74**(2): 553-561

236. König F., Krekeler G., Hönig J.F., Cordon-Cardo C., Fischer G. and Korabiowska M. Relation between human papillomavirus positivity and p16 expression in head and neck carcinomas - A tissue microarray study. *Anticancer Res* 2007; **27**(1A): 283-288

237. Koo C.-L., Kok L.-F., Lee M.-Y., Wu T.S., Cheng Y.-W., Hsu J.-D., Ruan A., Chao K.-C. and Han C.-P. Scoring mechanisms of $p16^{INK4a}$ immunohistochemistry based on either independent nucleic stain or mixed cytoplasmic with nucleic expression can significantly signal to distinguish between endocervical and endometrial adenocarcinomas in a tissue microarray study. *Journal of Translational Medicine* 2009; **7**:25

238. Koppikar P., De Villiers E.-M. and Mulherkar R. Identification of human papillomaviruses in tumors of the oral cavity in an Indian community. *Int J Cancer* 2005; **113**(6): 946-950

239. Kramer I.R.H., Lucas R.B. and Pindborg J.J. Definition of leukoplakia and related lesions: an aid to studies in precancer. *Oral Surg Oral Med and Oral Pathol* 1978; **46**(4): 517-539

240. Kreimer A.R., Clifford G.M., Boyle P. and Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidem Biomar* 2005; **14**(2): 467-475

241. Kreimer A.R., Clifford G.M., Snijders P.J.F., Castellsagué X., Meijer C.J.L.M., Pawlita M., Viscidi R., Herrero R. and Franceschi S. HPV16 semiquantitative

viral load and serologic biomarkers in oral and oropharyngeal squamous cell carcinomas. *Int J Cancer* 2005; **115**(2): 329-332

242. Kresty L.A., Mallery S.R., Knobloch T.J., Song H., Lloyd M., Casto B.C. and Weghorst C.M. Alterations of $p16^{INK4a}$ and $p14^{ARF}$ in Patients with Severe Oral Epithelial Dysplasia. *Cancer Res* 2002; **62**(18): 5295-5300

243. Kruk J. and Aboul-Enein H.Y. Environmental exposure and other behavioral risk factors in breast cancer. *Current Cancer Therapy Reviews* 2006; **2**(1): 3-21

244. Kulkarni V. and Saranath D. Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. *Oral Oncol* 2004; **40**(2): 145-153

245. Kumar B., Cordell K.G., Lee J.S., Worden F.P., Prince M.E., Tran H.H., Wolf G.T., Urba S.G., Chepeha D.B., Teknos T.N., Eisbruch A., Tsien C.I., Taylor J.M.G., D'Silva N.J., Yang K., Kurnit D.M., Bauer J.A., Bradford C.R. and Carey T.E. EGFR, p16, HPV Titer, Bcl-xL and p53, Sex, and Smoking As Indicators of Response to Therapy and Survival in Oropharyngeal. *Cancer J Clin Oncol* 2008; **26**(19): 3128-3137

246. Kune G.A. and McLaughlin S. Smoking, alcohol and squamous cell cancers of the oral cavity and gullet. *Med J Australia* 1983; **1**(5): 204

247. Kune G.A., Kune S., Field B., Watson L.F., Cleland H., Merenstein D. and Vitetta L. Oral and pharyngeal cancer, diet, smoking, alcohol, and serum vitamin A and β-carotene levels: A case-control study in men. *Nutr Cancer* 1993; **20**(1): 61-70

248. Kuo K., Hsiao C., Lin C., Kuo L., Huang S. and Lin M. The biomarkers of human papillomavirus infection in tonsillar squamous cell carcinoma - molecular basis and predicting favorable outcome. *Modern Pathol* 2008; **21**(4): 376-386

249. Kurkivuori J., Salaspuro V., Kaihovaara P., Kari K., Rautemaa R., Grönroos L., Meurman J.H. and Salaspuro M. Acetaldehyde production from ethanol by oral streptococci. *Oral Oncol* 2007; **43**(2): 181-186

250. Kurose K., Terai M., Soedarsono N., Rabello D., Nakajima Y., Burk R.D. and Takagi M. Low prevalence of HPV infection and its natural history in normal oral mucosa among volunteers on Miyako Island, Japan. *Oral Surg Oral Med O* 2004; **98**(1): 91-96

251. Kurumatani N., Kirita T., Zheng Y., Sugimura M. and Yonemasu K. Time trends in the mortality rates for tobacco- and alcohol-related cancers within the oral cavity and pharynx in Japan, 1950-94. *Journal of epidemiology/Japan Epidemiological Association* 1999; **9**(1): 46-52

252. Kuschnir H. Alcohol-nicotin: most often aetological factor of the oral-oropharyngeal cancer [ALKOHOL-NIKOTINABUSUS: HAUFIGSTER ATIOLOGISCHER FAKTOR DES MUNDHOHLEN-OROPHARYNX-MALIGNOMS]. *Arch Oto-Rhino-Laryn* 1980; **227**(3-4): 574-577

253. Lambert A.P.F., Anschau F. and Schmitt V.M. p16$^{INK4a}$ expression in cervical premalignant and malignant lesions. *Exp Mol Pathol* 2006; **80**(2): 192-196

254. Laco J., Slaninka I., Jirásek M., Čelakovský P., Vošmiková H. and Ryška A. High-risk human papillomavirus infection and p16$^{INK4a}$ protein expression in laryngeal lesions. *Pathol Res Pract* 2008; **204**(8): 545-552

255. Lajer C.B. and Buchwald C.V. The role of human papillomavirus in head and neck cancer. *APMIS* 2010; **118**(6-7): 510-519

256. Lakshmi S., Rema P. and Somanathan T. p16 is a surrogate marker for high-risk and malignant cervical lesions in the presence of human papillomavirus. *Pathobiology* 2009; **76**(3): 141-148

257. LaMere B.J., Howell R., Fetterman B., Shieh J. and Castle P.E. Impact of 6-month frozen storage of cervical specimens in alkaline buffer conditions on human papillomavirus genotyping. *J Virol Methods* 2008; **151**(2): 298-300

258. Lazarus P., Sheikh S.N., Ren Q., Schantz S.P., Stern J.C., Richie J.P.Jr and Park J.Y. p53, but not p16 mutations in oral squamous cell carcinomas are associated with specific CYP1A1 and GSTM1 polymorphic genotypes and patient tobacco use. *Carcinogenesis* 1998; **19**(3): 509-514

259. Lee J.K., Kim M.J., Hong S.P. and Hong S.D. Inactivation patterns of p16/INK4A in oral squamous cell carcinomas. *Exp Mol Med* 2004; **36**(2): 165-171

260. Lee H.S., Kim K.M., Kim S.M., Choi Y.D., Nam J.H., Park C.S. and Choi H.S. Human papillomavirus genotyping using HPV DNA chip analysis in Korean women. *Int J Gynecol Cancer* 2007; **17**(2): 497-501

261. Lee S.H., Vigliotti V.S., Vigliotti J.S. and Pappu S. Validation of human papillomavirus genotyping by signature DNA sequence analysis. *BMC Clinical Pathology* 2009; **9**(1): 3

262. Leedham S.J., Graham T.A., Oukrif D., McDonald S.A.C., Rodriguez-Justo M., Harrison R.F., Shepherd N.A., Novelli M.R., Jankowski J.A.Z. and Wright N.A. Clonality, Founder Mutations, and Field Cancerization in Human Ulcerative Colitis–Associated Neoplasia. *Gastroenterology* 2009; **136**(2): 542-550.e6

263. Letian T. and Tianyu Z. Cellular receptor binding and entry of human papillomavirus. *Virol J* 2010; **7**: 1-7

264. Liao C.T., Chang J.T., Wang H.M., Ng S.H., Hsueh C., Lee L.Y., Lin C.H., Chen I.H., Huang S.F., Cheng A.J. and Yen T.C. Analysis of risk factors of predictive local tumor control in oral cavity cancer. *Ann Surg Oncol* 2008; **15**(3): 915-22

265. Liao C.-T., Chen I.-H., Chang J.T.-C., Wang H.-M., Hsieh L.-L. and Cheng, A.-J. Lack of Correlation of Betel Nut Chewing, Tobacco Smoking, and Alcohol

Consumption with Telomerase Activity and the Severity of Oral Cancer. *Chang Gung Medical Journal* 2003; **26**(9): 637-645

266. Liggett W.H. and Sidransky D. Role of the p16 Tumor Suppressor Gene in Cancer. *J Clin Oncol* 1998; **16**(3): 1197-1206

267. Lillo F.B. and Uberti-Foppa C. Human papillomavirus viral load: a possible marker for cervical disease in HIV-infected women. *J Antimicrob Chemoth* 2006; **57**(5): 810-814

268. Lin S.C., Chang K.W., Chang C.S., Liu T.Y., Tzeng Y.S., Yang F.S. and Wong Y.K. Alterations of p16/MTS1 gene in oral squamous cell carcinomas from Taiwanese. *J Oral Pathol Med* 2000; **29**(4): 159-66

269. Lin C.Y., Chen H.C., Lin R.W., You S.L., You C.M., Chuang L.C., Pan M.H., Lee M.H., Chou Y.C. and Chen C.J. Quality assurance of genotyping array for detection and typing of human papillomavirus. *J Virol Methods* 2007; **140**(1-2): 1-9

270. Lin C.Y., Chao A., Yang Y.C., Chou H.H., Hoe C.M., Lin R.W., Chang T.C., Chiou J.Y., Chao F.Y., Wang K.L., Chien T.Y., Hsueh S., Huang C.C., Cheng C.J. and Lai C.H. Human papillomavirus typing with a polymerase chain reaction-based genotyping array compared with type-specific PCR. *J Clin Virol* 2008; **42**(4): 361-367

271. Lissowska J., Pilarska A., Pilarski P., Samolczyk-Wanyura D., Piekarczyk J., Bardin-Mikolajczak A., Zatonski W., Herrero R., Muňoz, N. and Franceschi S. Smoking, alcohol, diet, dentition and sexual practices in the epidemiology of oral cancer in Poland. *Eur J Cancer Prev* 2003; **12**(1): 25-33

272. Li J., Yang J., Yi Z., Lin Y. and Zhou A. Human papillomavirus infection and expression of p16 protein in laryngeal papilloma and laryngeal carcinoma. *Zhonghua er bi yan hou ke za zhi* 2001; **36**(1): 51-54

273. Li W., Thompson C.H., O'Brien C.J., McNeil E.B., Scolyer R.A., Cossart Y.E., Veness M.J., Walker D.M., Morgan G.J. and Rose B.R. Human papillomavirus positivity predicts favourable outcome for squamous carcinoma of the tonsil. *Int J Cancer* 2003; **106**(4): 553-558

274. Li W., Thompson C.H., Cossart Y.E., O'Brien C.J., McNeil E.B., Scolyer R.A. and Rose B.R. The expression of key cell cycle markers and presence of human papillomavirus in squamous cell carcinoma of the tonsil. *Head Neck* 2004; **26**(1): 1-9

275. Llamas-Martínez S., Esparza-Gómez G., Campo-Trapero J., Cancela-Rodríguez P., Bascones-Martínez A., Moreno-López L.A., García-Núñez J.A. and Cerero-Lapiedra R. Genotypic determination by PCR-RFLP of human papillomavirus in normal oral mucosa, oral leukoplakia and oral squamous cell carcinoma samples in Madrid (Spain). *Anticancer Res* 2008; **28**(6A): 3733-3741

276. Llewelyn J. and Mitchell R. Smoking, alcohol and oral cancer in South East Scotland: A 10-year experience. *Brit J Oral Max Surg* 1994; **32**(3): 146-152

277. Lohavanichbutr P., Houck J., Fan W., Yueh B., Mendez E., Futran N., Doody D.R., Upton M.P., Farwell D.G., Schwartz S.M., Zhao L.P. and Chen C. Genome wide gene expression profiles of HPV-positive and HPV-negative oropharyngeal cancer potential implications for treatment choices. *Arch Otolaryngol* 2009; **135**(2): 180-188

278. López M., Aguirre J.M., Cuevas N., Anzola M., Videgain J., Aguirregaviria J. and de Pancorbo M.M. Gene promoter hypermethylation in oral rinses of leukoplakia patients - a diagnostic and/or prognostic tool? *Eur J Cancer* 2003; **39**(16): 2306-2309

279. Lucenteforte E., Garavello W., Bossetti C. and La Vecchia C. Dietary factors and oral and pharyngeal cancer risk. *Oral Oncol* 2009; **45**(6): 461-467

280. Lu D.W., El-Mofty S.K. and Wang H.L. Expression of p16, Rb, and p53 proteins in squamous cell carcinomas of the anorectal region harboring human papillomavirus DNA. *Modern Pathol* 2003; **16**(7): 692-699

281. Luo C.-W., Roan C.-H. and Liu C.-J. Human papillomaviruses in oral squamous cell carcinoma and pre-cancerous lesions detected by PCR-based gene-chip array. *Int J Oral Max Surg* 2007; **36**(2): 153-158

282. Lydiatt W.M., Anderson P.E., Bazzana T., Casale M., Hughes C.J., Huvos A.G., Lydiatt D.D. and Schantz S.P. Molecular support for field cancerization in the head and neck. *Cancer* 1998; **82**(7): 1376-1380

283. Macfarlane G.J., Zheng T., Marshall J.R., Boffetta P., Niu S., Brasure J., Merletti F. and Boyle P. Alcohol, tobacco, diet and the risk of oral cancer: A pooled analysis of three case-control studies. *Eur J Cancer Part B: Oral Oncol* 1995; **31**(3): 181-187

284. Maier H., Tisch M., Conradt C. and Pötschke-Langer M. Alcohol consumption and cancer of the oral cavity, pharynx or larynx in women [Alkoholkonsum und krebs des oberen aerodigestivtraktes bei frauen]. *Deutsche Medizinische Wochenschrift*, 1999; **124**(28-29): 851-854

285. Man Y., Moinfar F., Bratthauer G.L., Kuhls E.A. and Tavassoli F.A. An improved method for DNA extraction from paraffin sections. *Pathol Res Pract* 2001; **197**(9): 635-642

286. Manos M.M., Ting Y., Wright D.K., Lewis A.J., Broker T.R. and Wolinsky S.M. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Molecular Diagnostics of Human Cancer: Cancer Cells* 1989; **7**: 209-214

287. Mao L., Hong W.K. and Papadimitrakopoulou V.A. Focus on head and neck cancer. *Cancer Cell* 2004; **5**(4): 311-316

288. Marais D.J., Sampson C., Jeftha A., Dhaya D., Passmore J.-A.S., Denny L., Rybicki E.P., van der Walt E., Stephen L.X.G. and Williamson A.-L. More men than women make mucosal IgA antibodies to human papillomavirus type 16 (HPV-16) and HPV-18: A study of oral HPV and oral HPV antibodies in a normal healthy population. *BMC Infect Dis* 2006; **6**: 95

289. Marichalar-Mendia X., Rodriguez-Tojo M.J., Acha-Sagredo A., Rey-Barja N. and Aguirre-Urizar J.M. Oral cancer and polymorphism of ethanol metabolising genes. *Oral Oncol* 2010; **46**(1): 9-13

290. Marks M., Gupta S.B., Liaw K-L, Kim E., Tadesse A., Coutlee F., Sriplienchan S., Celentano D.D. and Gravitt P.E. Confirmation and quantitation of human papillomavirus type 52 by Roche LinearArray© using HPV52-specific TaqMan© E6/E7 quantitative real-time PCR. *J Virol Methods* 2009; **156**(1-2): 152-156

291. Marshall J.R., Graham S., Haughey B.P., Shedd D., O'Shea R., Brasure J., Wilkinson G.S. and West D. Smoking, alcohol, dentition and diet in the epidemiology of oral cancer. *Eur J Cancer Part B: Oral Oncol* 1992; **28**(1): 9-15

292. Marur S., and Forastiere A.A. Head and neck cancer: Changing epidemiology, diagnosis and treatment. *Mayo Clin Proc* 2008; **83**(4): 489-501

293. Martins A.S. Multicentricity in pharyngoesophageal tumors: Argument for total pharyngolaryngoesophagectomy and gastric transposition. *Head Neck* 2000; **22**(2): 156–163

294. Mashberg A., Boffetta P., Winkelman R. and Garfinkel L. Tobacco smoking, alcohol drinking, and cancer of the oral cavity and oropharynx among U.S. Veterans. *Cancer* 1993; **72**(4): 1369-1375

295. Mashberg A. and Samit A. Early Diagnosis of Asymptomatic Oral and Oropharyngeal Squamous Cancers. *Cancer J Clin* 1995; **45**(6): 328-351

296. Massano J., Regateiro F.S., Januário G. and Ferreira A. Oral squamous cell carcinoma: Review of prognostic and predictive factors. *Oral Surg Oral Med O* 2006; **102**(1): 67-76

297. Massarrat S. Smoking and gut. *Archives of Iranian Medicine* 2008; **11**(3): 293-305

298. Matta A., Tripathi S.C., DeSouza L.V., Grigull J., Kaur J., Chauhan S.S., Srivastava A., Thakar A., Shukla N.K., Duggal R., DattaGupta S., Ralhan R. and Siu K.W.M. Heterogeneous ribonucleoprotein K is a marker of oral leukoplakia and correlates with poor prognosis of squamous cell carcinoma. *Int J Cancer* 2009; **125**(6): 1398-1406

299. Matthews K., Leong C.M., Baxter L., Inglis E., Yun K., Bäckström B.T., Doorbar J. and Hibma M. Depletion of Langerhans cells in human papillomavirus type 16-

infected skin is associated with E6-mediated down regulation of E-cadherin. *J Virol* 2003; **77**(15): 8378-85

300. Mayne S.T., Cartmel B., Kirsh V. and Goodwin Jr. W.J. Alcohol and tobacco use prediagnosis and postdiagnosis, and survival in a cohort of patients with early stage cancers of the oral cavity, pharynx, and larynx. *Cancer Epidem Biomar* 2009; **18**(12): 3368-3374

301. McBride A.A., Romanczuk H. And Howlwy P.M. The papillomavirus E2 regulatory proteins. *J Biol Chem* 1991; **266**(28): 18411-18414

302. McIntyre M.C., Ruesch M.N. and Laimins L.A. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. *Virology* 1996; **215**(1): 73-82

303. Meftah el khair M., El Mzibri M., Mhand R.A., Benider A., Benchekroun N., El fahime E.M., Benchekroun M.N. and Ennaji M.M. Molecular Detection and Genotyping of Human Papillomavirus in Cervical Carcinoma Biopsies in an Area of High Incidence of Cancer From Moroccan Women. *J Med Virol* 2009; **81**(4): 678-684

304. Melchers W.J.G., Bakkers J.M.J.E., Wang J., de Wilde P.C.M., Boonstra H., Quint W.G.V. and Hanselaar A.G.J.M. Short Fragment Polymerase Chain Reaction Reverse Hybridization Line Probe Assay to Detect and Genotype a Broad Spectrum of Human Papillomavirus Types. *Am J Pathol* 1999; **155**(5): 1474-1478

305. Mignogna M.D., Fedele S., Lo Russo L. and Lo Muzio L. Lack of public awareness toward alcohol consumption as risk factor for oral and pharyngeal cancers. *Prev Med* 2001; **33**(2I): 137-139

306. Mignogna M.D., Fedele S., Lo Russo L., Mignogna L., de Rosa G. and Porter S.R. Field cancerization in oral lichen planus. *EJSO* 2007; **33**(3): 383-389

307. Miller C.S. and Johnstone B.M. Human papillomavirus as a risk factor for oral squamous cell carcinoma: A meta-analysis, 1982-1997. *Oral Surg Oral Med O* 2001; **91**(6): 622-635

308. Mitra S., Banerjee S., Misra C., Singh R.K., Roy A., Sengupta A., Panda C.K. and Roychoudhury S. Interplay between human papilloma virus infection and p53 gene alterations in head and neck squamous cell carcinoma of an Indian patient population. *J Clin Pathol* 2007; **60**(9): 1040-1047

309. Mitsudomi T., Yatabe, Y., Koshikawa T., Hatooka S., Shinoda M., Suyama M., Sugiura T., Ogawa M. and Takahashi T. Mutations of the P53 tumor suppressor gene as clonal marker for multiple primary lung cancers. *J Thorac Cardiov Sur* 1997; **114**(3): 354-360

310. Mo L.Z., Monnier-Benoit S., Kantelip B., Petitjean A., Riethmuller D., Prétet J.L. and Mougin C. Comparison of AMPLICOR® and Hybrid Capture II® assays for

high risk HPV detection in normal and abnormal liquid-based cytology: Use of INNO-LiPA Genotyping assay to screen the discordant results. *J Clin Virol* 2008; **41**(2): 104-110

311. Moore S.R., Johnson N.W., Pierce A.M. and Wilson D.F. The epidemiology of mouth cancer: a review of global incidence. *Oral Dis* 2000; **6**(2): 65-74

312. Moreno-López L.A., Esparza-Gómez G.C., González-Navarro A., Cerero-Lapiedra R., González-Hernández M.J. and Domínguez-Rojas V. Risk of oral cancer associated with tobacco smoking, alcohol consumption and oral hygiene: A case-control study in Madrid, Spain. *Oral Oncol* 2000; **36**(2): 170-174

313. Morgan G.A., Lech N.L., Gloeckner G.W. and Barrett K.C. SPSS for Introductory Statistics: Use and Interpretation 2nd Edition. 2004; Lawrence Erlbaum Associates, Publishers

314. Morrow A. (2007) *http://www.omnimedicalsearch.com/conditions-diseases/ oral-cancer-types.html*

315. Mount D.W. Chapter 4, Multiple Sequence Alignment, in *Bioinformatics: Sequence and Genome Analysis*. 2001; Cold Spring Harbour Laboratory Press. pp 139-204

316. Mount D.W. Chapter 7, Database Searching for Similar Sequences, in *Bioinformatics: Sequence and Genome Analysis*. 2001; Cold Spring Harbour Laboratory Press. pp 281-335

317. Muirhead D.M., Hoffman H.T. and Robinson R.A. Correlation of clinicopathological features with immunohistochemical expression of cell cycle regulatory proteins p16 and retinoblastoma: Distinct association with keratinisation and differentiation in oral cavity squamous cell carcinoma. *J Clin Pathol* 2006; **59**(7): 711-715

318. Mulvany N.J., Allen D.G and Wilson S.M. Diagnostic Utility of $p16^{INK4a}$, a reappraisal of its use in cervical biopsies. *Pathology* 2008; **40**(4): 355-344

319. Munirajan A.K., Kannan K., Bhuvarahamurthy V., Ishida I., Fujinaga K., Tsuchida N. and Shanmugam G. The status of human papillomavirus and tumor suppressor genes p53 and p16 in carcinomas of uterine cervix from India. *Gynecol Oncol* 1998; **69**(3): 205-209

320. Muñoz N., Castellsagué X., de González A.B. and Gissmann L. Chapter 1: HPV in the etiology of human cancer. *Vaccine* 2006; **24**(S3): S3/1-S3/10

321. Munro B.H. *Statistical Methods for Health Care Research* 5th Edition. 2005; Lippincott Williams & Wilkins.

322. Murti P.R., Daftary D.K., Bhonsle R.B., Gupta P.C., Mehta F.S. and Pindborg J.J. Malignant potential of oral lichen planus: observations in 722 patients from India. *J. Oral Pathol* 1986; **15**(2): 71-77

323. Muwonge R., Ramadas K., Sankila R., Thara S., Thomas G., Vinoda J. and Sankaranarayanan R. Role of tobacco smoking, chewing and alcohol drinking in the risk of oral cancer in Trivandrum, India: A nested case-control design using incident cancer cases. *Oral Oncol* 2008; **44**(5): 446-454

324. Nagler R.M. Tissue and serum borne tumor markers of oral squamous cell carcinoma: present and forthcoming therapeutic roles. *Eur J Plast Surg* 2002; **25**(5): 235-242

325. Nagpal J.K and Das B.R. Oral cancer: Reviewing the present understanding of its molecular mechanism and exploring the future directions for its effective management. *Oral Oncol* 2003; **39**(3): 213-221

326. Nair U.J., Nair J., Mathew B. and Bartsch H. Glutathione S-transferase M1 and T1 null genotypes as risk factors for oral leukoplakia in ethnic Indian betel quid/tobacco chewers. *Carcinogenesis* 1999; **20**(5): 743-748

327. Nakahara Y., Shintani S., Mihara M., Ueyama Y. and Matsumura T. High frequency of homozygous deletion and methylation of p16$^{INK4A}$ gene in oral squamous cell carcinomas. *Cancer Lett* 2001; **163**(2): 221-228

328. Nakahara Y., Shintani S., Mihara M., Hino S. and Hamakawa H. Detection of p16 promoter methylation in the serum of oral cancer patients. *Int J Oral Max Surg* 2006; **35**(4): 362-365

329. Nazarenko I., Kobayashi L., Giles J., Fishman C., Chen G. and Lorincz A. A novel method of HPV genotyping using Hybrid Capture® sample preparation method combined with GP5+/6+ PCR and multiplex detection on Luminex® XMAP®. *J VirolMethods* 2008; **154**(1-2): 76-81

330. Nehls K., Vinokurova S., Schmidt D., Kommoss F., Reuschenbach M., Kisseljov F., Einenkel J., Doeberitz M.V. K. and Wentzensen N. p16 methylation does not affect protein expression in cervical carcinogenesis. *Eur J Cancer* 2008; **44**(16): 2496-2505

331. Nemes J.A., Deli L., Nemes Z. and Márton I.J. Expression of p16$^{INK4A}$, p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma. *Oral Surg Oral Med O* 2006; **102**(3): 344-352

332. Nemes J.A., Redl P., Boda R., Kiss C. and Márfon I.J. Oral cancer report from Northeastern Hungry. *Pathol Oncol Res* 2008; **14**(1): 185-192

333. Nichols A.C., Faquin W.C., Westra W.H., Mroz E.A., Begum S., Clark J.R. and Rocco J.W. HPV-16 infection predicts treatment outcome in oropharyngeal squamous cell carcinoma. *Otolaryng Head Neck* 2009; **140**(2): 228-234

334. Nielsen A., Kjaer S.K., Munk C. and Iftner T. Type-specific HPV infection and multiple HPV types: Prevalence and risk factor profile in nearly 12,000 younger and older Danish women. *Sex Transm Dis* 2008; **35**(3): 276-282

335. Nobre R.J., de Almeida L.P. and Martins T.C. Complete genotyping of mucosal human papillomavirus using a restriction fragment length polymorphism analysis and an original typing algorithm. *J Clin Virol* 2008; **42**(1): 13-21

336. Nomenclature Committee of the International Union of Biochemistry (NC-IUB). Nomenclature for incompletely specified bases in nucleic acid sequences. Recommendations 1984. *Eur. J. Biochem.* 1985; **150**(1): 1-5

337. ÓFlatharta C., Flint S.R., Toner M., Butler D. and Mabruk, M.J.E.M.F. Investigation into a Possible Association between Oral Lichen Planus, the Human Herpesviruses, and the Human Papillomaviruses. *Mol Diagn* 2003; **7**(2): 73-83

338. Ogden G.R., Chisholm D.M., Morris A.M. and Stevenson J.H. Overexpression of p53 in normal oral mucosa of oral cancer patients does not necessarily predict further malignant disease. *J Pathol* 1997; **182**(2): 180-184

339. Ogden G.R. Field cancerisation in the head and neck. *Oral Dis* 1998; **4**(1): 1-3

340. Ogden G.R. and Wight A.J. Aetiology of oral cancer: Alcohol. *Brit J Oral Max Surg* 1998; **36**(4): 247-251

341. Ogden G.R. Alcohol and oral cancer. *Alcohol* 2005; **35**(3): 169-173

342. Ohta S., Uemura H., Matsui Y., Ishiguro H., Fujinami K., Kondo K., Miyamoto H., Yazawa T., Danenberg K., Danenberg P.V., Tohnai I. and Kubota Y. Alterations of p16 and $p14^{ARF}$ genes and their 9p21 locus in oral squamous cell carcinoma. *Oral Surg Oral Med O* 2009; **107**(1): 81-91

343. Ota Y., Aoki T., Karakida K., Otsuru M., Kurabayashi H., Sasaki M., Nakamura N. and Kajiwara H. Determination of deep surgical margin based on anatomical architecture for local control of squamous cell carcinoma of the buccal mucosa. *Oral Oncol* 2009; **45**(7): 605-609

344. Ozgul N., Cil A.P., Bozdayi G., Usubutun A., Bulbul D., Rota S., Kose1 M.F., Biri A. and Haberal A. Staining characteristics of p16INK4a: Is there a correlation with lesion grade or high-risk human papilloma virus positivity? *J Obstet Gynaecol Res* 2008; **34**(5): 865-871

345. Palmer S. Diet, nutrition, and cancer. *Progress in food & nutrition science* 1985; **9**(3-4): 283-341

346. Park K., Ellenson L.H. and Pirog E.C. Low-grade squamous intraepithelial lesions of the cervix with marked cytological atypia - Clinical follow-up and human papillomavirus genotyping International. *Int J Gynecol Pathol* 2007; **26**(4): 457-462

347. Parsons B.L. Many different tumor types have polyclonal tumor origin: Evidence and implications. *Mutat Res* 2008; **659**(3): 232-247

348. Partridge M., Emilion G., Pateromichelakis S., Phillips E. and Langdon J. Field cancerization of the oral cavity: comparison of the spectrum of molecular alterations in cases presenting with both dysplastic and malignant lesions. *Oral Oncol* 1997; **33**(5): 332-337

349. Partridge M., Emilion G., Pateromichelakis S., A'Hern R., Phillips E. and Langdon J. Allelic imbalance at chromosomal loci implicated in the pathogenesis of oral precancer, cumulative loss and its relationship with progression to cancer. *Oral Oncol* 1998; **34**(2): 77-83

350. Partridge M., Emilion G., Pateromichelakis S., Phillips E. and Langdon J. Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas. *Int J Cancer* 1999; **83**(3): 318-325

351. Partridge M. Oral cancer: 1. The genetic basis of the disease. *Dental Update* 2000; **27**(5): 242-248

352. Partridge M. Oral cancer: 2. Clinical presentation and use of new knowledge about the biology of cancer to establish why tumors may recur. *Dental Update* 2000; **27**(6): 288-294

353. Partridge M., Pateromichelakis S., Phillips E., Emilion G., and Langdon J. Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma. *Clin Cancer Res* 2001; **7**(7): 1860-1866

354. Patel S.G. and Shah J.P. TNM staging of cancers of the head and neck: Striving for uniformity among diversity. *Ca-A Cancer J. Clin* 2005; **55**(4): 242-258

355. Pateromichelakis S., Farahani M., Phillips E., and Partridge M. Molecular analysis of paired tumours: Time to start treating the field. *Oral Oncol* 2005; **41**(9): 916-926

356. Patiman, Zhang Z. and Cao J. Research on expression of human papillomavirus type 16 and telomerase in oral lesions. Zhonghua kou qiang yi xue za zhi = Zhonghua kouqiang yixue zazhi = *Chinese Journal of Stomatology* 2001; **36**(2): 119-121

357. Penning T.M. and Lerman C. Genomics of smoking exposure and cessation: Lessons for cancer prevention and treatment. *Cancer Prev Res* 2008; **1**(2): 80-83

358. Pentenero M., Gandolfo S. and Carrozzo M. Importance of tumour thickness and depth of invasion in nodal involvement and prognosis of oral squamous cell carcinoma: A review of the literature. *Head Neck* 2005; **27**(12): 1080-1091

359. Peters E.S., McClean M.D., Liu M., Eisen E.A., Mueller N. and Kelsey K.T. The ADH1C polymorphism modifies the risk of squamous cell carcinoma of the head and neck associated with alcohol and tobacco use. *Cancer Epidem Biomar* 2005; **14**(2): 476–82

360. Petersen P.E. Oral cancer prevention and control - The approach of the World Health Organization. *Oral Oncol* 2009; **45**(4-5): 454-460

361. Petti L, Nilson L.A. and DiMaio D. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO J* 1991; **10**(4): 845-855

362. Petti L. and DiMaio D. Specific interaction between the bovine papillomavirus E5 transforming protein and the β receptor for platelet-derived growth factor in stably transformed and acutely transfected cells. *J Virol* 1994; **68**(6): 3582-3592

363. Petti S. and Scully C. Oral cancer: The association between nation-based alcohol-drinking profiles and oral cancer mortality. *Oral Oncol* 2005; **41**(8): 828-834

364. Petti S. Lifestyle risk factors for oral cancer. *Oral Oncol* 2009; **45**(4-5): 340-350

365. Pfister H. Chapter 8: Human papillomavirus and skin cancer. *J Natl Cancer Inst Monogr* 2003; (31): 52-56

366. Pim D. and Banks L. Interaction of viral oncoproteins with cellular target molecules: infection with high-risk vs low-risk human papilomavirus. *APMIS* 2010; **118**(6-7): 471-493

367. Pitiyage G., Tilakaratne W.M., Tavassoli M. and Warnakulasuriya S. Molecular markers in oral epithelial dysplasia: review. *J Oral Pathol Med* 2009; **38**(10): 737-752

368. Pittayakhajonwut D. and Angeletti P.C. Viral trans-factor independent replication of human papillomavirus genomes. *Virol J* 2010; **7**: 123

369. Poblet E., Pascual A., Godánez J.M., Pariente-Martín M., Escario E. and Garcıá-Olmo D.C. Human papillomavirus-associated penile sarcomatoid carcinoma. *J Cutan Pathol* 2008; **35**(6): 559-565

370. Pöschl G. and Seitz H.K. Alcohol and cancer. *Alcohol Alcoholism* 2004; **39**(3): 155-165

371. Preuss S.F., Weinell A., Molitor M., Stenner M., Semrau R., Drebber U., Weissenborn S.J., Speel E.J.M., Wittekindt C., Guntinas-Lichius O., Hoffmann T.K., Eslick G.D. and Klussmann J.P. Nuclear survivin expression is associated with HPV-independent carcinogenesis and is an indicator of poor prognosis in oropharyngeal cancer. *Brit J Cancer* 2008; **98**(3): 627-632

372. Prime S.S., Eveson J.W., Guest P.G., Parkinson E.K. and Paterson I.C. Early genetic and functional events in the pathogenesis of oral cancer. *Radiat Oncol Investi* 1997; **5**(3): 93-96

373. Purdie K.J., Sexton C.J., Proby C.M., Glover M.T., Williams A.T., Stables J.N. and Leigh I.M. Malignant transformation of cutaneous lesions in renal allograft patients: A role for human papillomavirus? *Cancer Res* 1993; **53**(21): 5328-5333

374. Purdie K.J., Surentheran T., Sterling J.C., Bell L., McGregor J.M., Proby C.M., Harwood C.A. and Breuer J. Human papillomavirus gene expression in cutaneous squamous cell carcinomas from immunosuppressed and immunocompetent individuals. *J Invest Dermatol* 2005; **125**(1): 98-107

375. Ragin C.C.R. and Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: Review and meta-analysis. *Int J Cancer* 2007; **121**(8): 1813-1820

376. Raimondi A., Cabrini R. and Itoiz M.E. Ploidy analysis of field cancerization and cancer development in the hamster cheek pouch carcinogenesis model. *J Oral Pathol Med* 2005; **34**(4): 227-231

377. Ralhan R., DeSouza L.V., Matta A., Tripathi S.C., Ghanny S., DattaGupta S., Thakar A., Chauhan S.S. and Siu K.W.M. iTRAQ-Multidimensional Liquid Chromatography and Tandem Mass Spectrometry-Based Identification of Potential Biomarkers of Oral Epithelial Dysplasia and Novel Networks between Inflammation and Premalignancy. *J Proteome Res* 2009; **8**(1): 300-309

378. Rao D.N. and Desai P.B. Risk assessment of tobacco, alcohol and diet in cancers of base tongue and oral tongue - A case control study. *Indian J Cancer* 1998; **35**(2): 65-72

379. Rao D.N., Ganesh B., Rao R.S. and Desai P.B. Risk assessment of tobacco, alcohol and diet in oral cancer. A case-control study. *Int J Cancer* 1994; **58**(4): 469-473

380. Rapidis A.D., Gullane P., Langdon J.D., Lefebre J.L., Scully C. and Shah J.P. Major advances in the knowledge and understanding of the epidemiology, aetiopathogenesis, diagnosis, management and prognosis of oral cancer. *Oral Oncol* 2009; **45**(4-5): 299-300

381. Reibel J. Significance of clinical, histopathological, and molecular biological characteristics. *Crit Rev Oral Biol Med* 2003; **14**(1): 47-62

382. Reimers N., Kasper H.U., Weissenborn S.J., Stützer H., Preuss S.F., Hoffmann T.K., Speel E.J.M., Dienes H.P., Pfister H.J., Guntinas-Lichius O. and Klussmann J.P. Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. *Int J Cancer* 2007; **120**(8): 1731-1738

383. Roberts S., Ashmole I., Johnson G.D., Kreider J.W. and Gallimore P.H. Cutaneous and mucosal human papillomavirus E4 proteins form intermediate filament-like structures in epithelial cells. *Virology* 1993; **197**(1): 176-187

384. Rocco J.W. and Sidransky D. p16(MTS-1/CDKN2/INK4a) in cancer progression. *Exp Cell Res* 2001; **264**(1): 42-55

385. Rodolico V., Barresi E., Di Lorenzo R., Leonardi V., Napoli P., Rappa F. and Di Bernardo C. Lymph node metastasis in lower lip squamous cell carcinoma in

relation to tumour size, histologic variables and p27Kip1 protein expression. *Oral Oncol* 2004; **40**(1): 92-98

386. Rodu B. and Godshall W.T. Tobacco harm reduction: an alternative cessation strategy for inveterate smokers. *Harm Red J* 2006; **3**(37): 1-23

387. Rodu B. and Jansson C. Smokeless tobacco and oral cancer: a review of the risks and determinants. *Crit Rev Oral Biol Med* 2004; **15**(5): 252-263

388. Rodu B. and Phillips C.V. The Association of Nation-Based Alcohol-Drinking Profiles and Oral Cancer Mortality Remains Unclear. *J Evid Base Dent Pract* 2007; **7**(2): 75-76

389. Rogers S.N., Brown J.S., Woolgar J.A., Lowe D., Magennis P., Shaw R.J., Sutton D., Errington D. and Vaughan D. Survival following primary surgery for oral cancer. *Oral Oncol* 2009; **45**(3): 201-211

390. Rosas S.L.B., Koch W., Da Costa Carvalho M.D.G., Wu L., Califano J., Westra W., Jen J. and Sidransky D. Promoter hypermethylation patterns of p16, $O^6$-methylguanine-DNA methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. *Cancer Res* 2001; **61**(3): 939-942

391. Ruas M. and Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998; **1378**(2): F115-177

392. Ruesga M.T., Acha-Sagredo A., Rodríguez M.J., Aguirregaviria J.I., Videgain J., Rodríguez C., de Pancorbo M.D.L.A.M. and Aguirre J.M. p16INK4a promoter hypermethylation in oral scrapings of oral squamous cell carcinoma risk patients. *Cancer Lett* 2007; **250**(1): 140-145

393. Saatci C., Caglayan A.O., Ozkul Y., Tahiri S., Turhan A.B. and Dundar M. Detection of p16 promotor hypermethylation in ‘‘Maras powder’’ and tobacco users. *Cancer Epidem* 2009; **33**(1): 47-50

394. Sabol I., Salakova M., Smahelova J., Pawlita M., Schmitt M., Gašperov N.M., Grce M. and Tachezy R. Evaluation of Different Techniques for Identification of Human Papillomavirus Types of Low Prevalence. *J Clin Microbiol* 2008; **46**(5): 1606-1613

395. Sailasree R., Abhilash A., Sathyan K.M., Nalinakumari K.R., Thomas S. and Kannan S. Differential Roles of $p16^{INK4A}$ and $p14^{ARF}$ Genes in Prognosis of Oral Carcinoma. *Cancer Epidem Biomar* 2008; **17**(2): 414-20

396. Salaspuro M.P. Alcohol consumption and cancer of the gastrointestinal tract. *Best Pract Res Cl Ga* 2003; **17**(4): 679-694

397. Salehi F., Turner M.C., Phillips K.P., Wigle D.T., Krewski D. and Aronson K.J. Review of the etiology of breast cancer with special attention to organochlorines as potential endocrine disruptors. *J Toxicol Env Heal B* 2008; **11**(3-4): 276-300

398. Salem A. Dismissing links between HPV and aggressive tongue cancer in young patients. *Ann Oncol* 2009; **21**(1): 13-17

399. Samama B., Lipsker D. and Boehm N. p16 expression in relation to human papillomavirus in anogenital lesions. *Hum Pathol* 2006; **37**(5): 513-519

400. Samama B., Schaeffer C. and Boehm N. p16 expression in relation to human papillomavirus in liquid-based cervical smears. *Gynecol Oncol* 2008; **109**(2): 285-290

401. Sanderson R.J., De Boer M.F., Damhuis R.A.M., Meeuwis C.A. and Knegt P.P. The influence of alcohol and smoking on the incidence of oral and oropharyngeal cancer in women. *Clin Otolaryngol Allied Sci* 1997; **22**(5): 444-448

402. Sano T., Oyama T., Kashiwabara K., Fukuda T. and Nakajima T. Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *Am J Pathol* 1998; **153**(6): 1741-1748

403. Sano T., Masuda N., Oyama T. and Nakajima T. Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. *Pathol Int* 2002; **52**(5-6): 375-383

404. Sarver N., Robson M.S. and Yang Y.C. Localization and analysis of bovine papillomavirus type 1 transforming functions. *J Virol* 1984; **52**(2): 377-388

405. Sathyan K.M., Sailasree R., Jayasurya R., Lakshminarayanan K., Abraham T., Nalinakumari K.R., Abraham E.K. and Kannan S. Carcinoma of tongue and the buccal mucosa represent different biological subentities of the oral carcinoma. *J Cancer Res Clin Oncol* 2006; **132**(9): 601–609

406. Sato Y., Sugie R., Tsuchiya B., Kameya T., Natori M. and Mukai K. Comparison of the DNA extraction methods for polymerase chain reaction amplification from formalin-fixed and paraffin-embedded tissues. *Diagn Mol Pathol* 2001; **10**(4): 265-271

407. Schafer K.A. The Cell Cycle: A Review. *Vet Pathol* 1998; **35**(6): 461-478

408. Schenk T., Brandstetter T., Zur Hausen A., Alt-Mörbe J., Huzly D. and Rühe J. Performance of a Polymer-Based DNA Chip Platform in Detection and Genotyping of Human Papillomavirus in Clinical Samples. *J Clin Microbiol* 2009; **47**(5): 1428-1435

409. Schildt E.-B., Eriksson M., Hardell L. and Magnuson A. Oral SNUFF, smoking habits and alcohol consumption in relation to oral cancer in a Swedish case-control study. *Int J Cancer* 1998; **77**(3): 341-346

410. Schlecht N.F., Burk R.D., Adrien L., Dunne A., Kawachi N., Sarta C., Chen Q., Brandwein-Gensler M., Prystowsky M.B., Childs G., Smith R.V. and Belbin T.J.

Gene expression profiles in HPV-infected head and neck cancer. *J Pathol* 2007; **213**(3): 283–293

411. Schliephake H. Prognostic relevance of molecular markers of oral cancer - A review. *Int J Oral Max Surg* 2003; **32**(3): 233–245

412. Schmitt M., Dondog B., Waterboer T., and Pawlita M. Homogeneous amplification of genital human alpha papillomaviruses by PCR using novel broad-spectrum GP5+ and GP6+ primers. *J Clin Microbiol* 2008; **46**(3): 1050-1059

413. Scholes A.G.M., Woolgar J.A., Boyle M.A., Brown J.S., Vaughan E.D., Hart C.A., Jones A.S., and Field J.K. Synchronous oral carcinomas: Independent or common clonal origin? *Cancer Res* 1998; **58**(9): 2003-2006

414. Schwartz G.K. and Shah M.A. Targeting the cell cycle: A new approach to cancer therapy. *J Clin Oncol* 2005; **23**(36): 9408-9421

415. Schwartz S.M., Doody D.R., Dawn Fitzgibbons E., Ricks S., Porter P.L. and Chen C. Oral squamous cell cancer risk in relation to alcohol consumption and alcohol dehydrogenase-3 genotypes. *Cancer Epidem Biomar* 2001; **10**(11): 1137-1144

416. Schwartz S.R., Yueh B., McDougall J.K., Daling J.R. and Schwartz S.M. Human papillomavirus infection and survival in oral squamous cell cancer: A population-based study. *Otolaryng Head Neck* 2001; **125**(1): 1-9

417. Scully C., Field J.K. and Tanzawa H. Genetic aberrations in oral or head and neck squamous cell carcinoma 2: chromosomal aberrations. *Oral Oncol* 2000; **36**(4): 311-327

418. Scully C., Sudbø J. and Speight P.M. Progress in determining the malignant potential of oral lesions. *J Oral Pathol Med* 2003; **32**(5): 251-256

419. Scully C. and Boyle P. The role of the Dental Team in Preventing and Diagnosing Cancer: 1. Cancer in General. *Dental Update* 2005; **32**(4): 204-212

420. Scully C., Newman L. and Bagán J.V. The role of the Dental Team in Preventing and Diagnosing Cancer: 2. Oral Cancer Risk Factors. *Dental Update* 2005; **32**(5): 261-276

421. Scully C. and Bagán J.V. Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. *Oral Dis* 2009; **15**(6): 388-399

422. Scully C. and Bagán J.V. Recent advances in oral oncology 2008; squamous cell carcinoma imaging, treatment, prognostication and treatment outcomes. *Oral Oncol* 2009; **45**(6): e25-e30

423. Scully C. and Bagán J.V. Oral squamous cell carcinoma overview. *Oral Oncol* 2009; **45**(4-5): 301-308

424. Serrano M., Hannon G.J. and Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993; **366**(6456): 704-707

425. Seme K., Lepej S.Z., Lunar M.M., Iščić-Beš J., Planinić A., Kocjan B.J., Vince A. and Poljak M. Digene HPV Genotyping RH Test RUO: Comparative evaluation with INNO-LiPA HPV Genotyping Extra Test for detection of 18 high-risk and probable high-risk human papillomavirus genotypes. *J Clin Virol* 2009; **46**(2): 176–179

426. Shafer W.G. and Waldron C.A. Erythroplakia of the oral cavity. *Cancer* 1975; **36**(3): 1021-1028

427. Shah N.G., Trivedi T.I., Tanskhali R.A., Goswami J.V., Jetly D.H., Shukla S.N., Shah P.M. and Verma R.J. Prognostic significance of molecular markers in oral squamous cell carcinoma: A multivariate analysis. *Head Neck* 2009; **31**(12): 1544-1556.

428. Shah J.P. and Gil Z. Current concepts in management of oral cancer - Surgery. *Oral Oncol* 2009; **45**(4-5): 394-401

429. Shaw R.J., Liloglou T., Rogers S.N., Brown J.S., Vaughan E.D., Lowe D., Field J.K. and Risk J.M., Promoter methylation of P16, RARβ, E-cadherin, cyclin A1 and cytoglobin in oral cancer: Quantitative evaluation using pyrosequencing. *Brit J Cancer* 2006; **94**(4): 561-568

430. Shaw R.J., Hall G.L., Woolgar J.A., Lowe D., Rogers S.N., Field J.K., Liloglou T. and Risk J.M. Quantitative methylation analysis of resection margins and lymph nodes in oral squamous cell carcinoma. *Brit J Oral Max Surg* 2007; **45**(8): 617-622

431. Shaw R.J., McGlashan G., Woolgar J.A., Lowe D., Brown J.S., Vaughan E.D., and Rogers S.N. Prognostic importance of site in squamous cell carcinoma of the buccal mucosa. *Brit J Oral Max Surg* 2009; **47**(5): 356-359

432. Sherman L., Jackman A., Itzhaki H., Stoppler M.C. Koval D. and Schlegel R. Inhibition of serum- and calcium-induced differentiation of human keratinocytes by HPV16 E6 oncoprotein: Role of p53 inactivation. *Virology* 1997; **237**(2): 296-306

433. Shillitoe E.J. The role of viruses in squamous cell carcinoma of the oropharyngeal mucosa. *Oral Oncol* 2009; **45**(4-5): 351-355

434. Shintani S., Nakahara Y., Mihara M., Ueyama Y. And Matsumura T. Inactivation of the $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ genes is a frequent event in human oral squamous cell carcinomas. *Oral Oncol* 2001; **37**(6): 498-504

435. Shintani S., Mihara M., Nakahara Y., Kiyota A., Ueyama Y., Matsumura T. and Wong D.T.W. Expression of cell cycle control proteins in normal epithelium,

premalignant and malignant lesions of oral cavity. *Oral Oncol* 2002; **38**(3): 235-243

436. Si H.X., Tsao S.W., Poon C.S.P., Wong Y.C. and Cheung A.L.M. Physical status of HPV-16 in esophageal squamous cell carcinoma. *J Clin Virol* 2005; **32**(1): 19-23

437. Sieczka E., Datta R., Singh A., Loree T., Rigual N., Orner J. and Hicks W Jr. Cancer of the buccal mucosa: are margins and T-stage accurate predictors of local control? *Am J Otolaryngol* 2001; **22**(6): 395-399

438. Simonato L.E., Garcia J.F., Nunes C.M. and Miyahara G.I. Evaluation of two methods of DNA extraction from paraffin-embedded material for PCR amplification. *J Bras Patol Med Lab* 2007; **43**(2): 121-127

439. Singhi A.D. and Westra W.H. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. *Cancer* 2010; **116**(9): 2166-2173

440. Sinha P., Bahadur S., Thakar A., Matta A., Macha M., Ralhan R. and Gupta S.D. Significance of promoter hypermethylation of p16 gene for margin assessment in carcinoma tongue. *Head Neck* 2009; **31**(11): 1423-1430

441. Slaughter D. P., Southwick H. W., and Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical. *Cancer* 1953; **6**(5): 963-968

442. Smeets S.J., Hesselink A.T., Speel E.M., Haesevoets A., Snijders P.J.F., Pawlita M., Meijer C.J.L.M, Braakhuis B.J.M., Leemans C.R. and Brakenhoff R.H. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer* 2007; **121**(1): 2465-2472

443. Smith I.M., Mydlarz W.K., Mithani S.K. and Califano J.A. DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. *Int J Cancer* 2007; **121**(8): 1724-1728

444. Smith E.M., Ritchie J.M., Pawlita M., Rubenstein L.M., Haugen T.H., Turek L.P. and Hamsikova E. Human papillomavirus seropositivity and risks of head and neck cancer. *Int J Cancer* 2007; **120**(4): 825-832

445. Smith E.M., Wang D., Kim Y., Rubenstein L.M., Lee J.H., Haugen T.H. and Turek L.P. $p16^{INK4a}$ expression, human papillomavirus, and survival in head and neck cancer. *Oral Oncol* 2008; **44**(2): 133-142

446. Smith E.M., Rubenstein L.M., Hoffman H., Haugen T.H. and Turek L.P. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. *Infectious Agents and Cancer* 2010; **5**(1): 4

447. Snijders P.J.F., Van Den Brule A.J.C., Schrijnemakers H.F.J., Snow Meijer G.C.J.L.M. and Walboomers J.M.M. The use of general primers in the

polymerase chain reaction permits the detection of broad spectrum papillomavirus genotypes *J Gen Virol* 1990; **71**(1): 173-181

448. Snijders P.J.F., Scholes A.G.M. and Hart C.A. Prevalence of mucosatropic human papillomaviruses in squamous cell carcinomas of the head and neck. *Int J Cancer* 1996; **66**(4): 464-469

449. Snijders P.J.F., Heideman D.A.M. and Meijer C.J.L.M. Methods for HPV detection in exfoliated cell and tissue specimen. *APMIS* 2010; **118**(6-7): 520-528

450. Sparkowski J., Mense M., Anders J. and Schlegel R. E5 oncoprotein transmembrane mutants dissociate fibroblast transforming activity from 16-kilodalton protein binding and platelet-derived growth factor receptor binding and phosphorylation. *J Virol* 1996; **70**(4): 2420-2430

451. Spiro R.H., Huvos A.G., Wong G.Y., Spiro J.D., Gnecco C.A. and Strong E.W. Predictive value of tumor thickness in squamous carcinoma confined to the tongue and floor of mouth. *Am J Surg* 1986; **152**(4): 345-350

452. Stanley M., Lowry D.R. and Frazer I. Chapter 12: Prophylactic HPV vaccines: Underlying mechanisms. *Vaccine* 2006; **24**(S3): S3/106-S3/113

453. Stanley M. Immunobiology of HPV and HPV vaccines. *Gynecol Oncol* 2008; **109**(S2): S15-S21

454. Steele C. and Shillitoe E.J. Viruses and Oral Cancer. *Crit Rev Oral Biol M* 1991; **2**(2): 153-175

455. Steinberg B. and Norrild B. HPV-oral, pharyngeal and laryngeal infections *APMIS* 2010; **118**(6-7): 421

456. Stevens M.P., Garland S.M., and Tabrizi S.N. Validation of an automated detection platform for use with the Roche Linear Array human papillomavirus genotyping test. *J Clin Microbiol* 2008; **46**(11): 3813-3816

457. Sugiyama M., Bhawal U.K., Dohmen T., Ono S., Miyauchi M. and Ishikawa, T. Detection of human papillomavirus-16 and HPV-18 DNA in normal, dysplastic, and malignant oral epithelium. *Oral Surg Oral Med O* 2003; **95**(5): 594-600

458. Šupić, G., Kozomara, R., Branković-Magić, M., Jović, N. and Magić, Z. Gene hypermethylation in tumor tissue of advanced oral squamous cell carcinoma patients. *Oral Oncol* 2009; **45**(12): 1051-1057

459. Sutton D.N., Brown J.S., Rogers S.N., Vaughan E.D. and Woolgar J.A. The prognostic implications of the surgical margin in oral squamous cell carcinoma. *Int J Oral Max Surg* 2003; **32**(1): 30-34

460. Surentheran T., Harwood C.A., Spink P.J., Sinclair A.L., Leigh I.M., Proby C.M., McGregor J.M. and Breuer J. Detection and typing of human papillomaviruses in mucosal and cutaneous biopsies from immunosuppressed and immunocompetent

patients and patients with epidermodysplasia verruciformis: a unified diagnosis approach. *J Clin Pathol* 1998; **51**(8): 606-610

461. Suzuki H., Sugimura H. and Hashimoto K. p16$^{INK4A}$ in oral squamous cell carcinomas - A correlation with biological behaviors: Immunohistochemical and FISH analysis. *J Oral Maxil Surg* 2006; **64**(11): 1617-1623

462. Swan D.C., Tucker R.A., Tortolero-Luna G., Mitchell M.F., Wideroff L., Unger E.R., Nisenbaum R.A., Reeves W.C. and Icenogle J.P. Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV Type. *J Clin Microbiol* 1999; **37**(4): 1030-1034

463. Syrjänen K., Syrjänen S. and Lamberg M. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. *Int. J. Oral Surg* 1983; **12**(6): 418-424

464. Syrjänen K. and Syrjänen S. 4 Diagnostic Techniques in HPV Detection. In: *Papillomavirus Infections in Human Pathology*. 1999; John Wiley & Sons, Ltd

465. Syrjänen S. Human papillomavirus infections and oral tumors. *Med Microbiol Immunol* 2003; **192**(3): 123-128

466. Syrjänen S. Human papillomavirus (HPV) in head and neck cancer. *J Clin Virol* 2005; **32S**: S59–S66

467. Syrjänen S. Curent concepts on human papillomavirus infections in children. *APMIS* 2010; **118**(6-7): 494-509

468. Szabó G., Klenk G. and Veér A. Correlation between the combination of alcohol consumption and smoking in oral cancer (screening of the population at risk) [A krónikus alkoholfogyasztás és a dohányzás együttes összefüggése a szájüregi rákbetegséggel (szürövizsgálat a veszélyeztett populációban).] *Orvosi hetilap* 1997; **138**(52): 3297-3299

469. Tabor M.P., Brakenhoff R.H., van Houten V.M.M, Kummer J.A., Snel M.H.J., Snijders P.J.F., Snow G.B., Leemans C.R. and Braakhuis B.J.M. Persistence of genetically altered fields in head and neck cancer patients: Biological and clinical implications. *Clin Cancer Res* 2001; **7**(6): 1523-1532

470. Tadokoro K., Yamaguchi T., Egashira T. and Hara T. Quantitation of viral load by real-time PCR-monitoring Invader reaction. *J Virol Methods* 2009; **155**(2): 182-186

471. Takács T., Jeney C., Kovács L., Mózes J., Benczik M. and Sebe A. Molecular beacon-based real-time PCR method for detection of 15 high-risk and 5 low-risk HPV types. *J Virol Methods* 2008; **149**(1): 153-162

472. Takeshima M., Saitoh M., Kusano K., Nagayasu H., Kurashige Y., Malsantha M., Arakawa T., Takuma T., Chiba I., Kaku T., Shibata T. and Abiko Y. High

frequency of hypermethylation of p14, p15 and p16 in oral pre-cancerous lesions associated with betel-quid chewing in Sri Lanka. *J Oral Pathol Med* 2008; **37**(8): 475-479

473. Takezaki T., Hirose K., Inoue M., Hamajima N., Kuroishi T., Nakamura S., Koshikawa T., Matsuura H. and Tajima K. Tobacco, alcohol and dietary factors associated with the risk of oral cancer among Japanese. *Jpn J Cancer Res* 1996; **87**(6): 555-562

474. Talamini R., Franceschi S., Barra S. and La Vecchia C. The role of alcohol in oral and pharyngeal cancer in non-smokers, and of tobacco in non-drinkers. *Int J Cancer* 1990; **46**(3): 391-393

475. Talamini R., La Vecchia C., Levi F., Conti E., Favero A. and Franceschi S. Cancer of the oral cavity and pharynx in nonsmokers who drink alcohol and in nondrinkers who smoke tobacco. *J Natl Cancer I* 1998; **90**(24): 1901-1903

476. Tanaka R., Wang D., Morishita Y., Inadome Y., Minami Y., Iijima T., Fukai S., Goya T. And Noguchi M. Loss of function of p16 gene and prognosis of pulmonary adenocarcinoma. *Cancer* 2005; **103**(3): 608-615

477. Terai M., Hashimoto K., Yoda K. And Sata T. High prevalence of human papillomaviruses in the normal oral cavity of adults. *Oral Microbiol Immun* 1999; **14**(4): 201-205

478. Teschke R., Hennermann K.-H., Poschmann G. and Burk M. *Alcohol and cancer* [Alkohol und krebs]Verdauungskrankheiten 2001; **19**(1): 19-31

479. Thomas G., Hashibe M., Jacob B.J., Ramadas K., Mathew B., Sankaranarayanan R. and Zhang Z. Risk factors for multiple oral premalignant lesions. *Int J Cancer* 2003; **107**(2): 285-291

480. Thomas S.J., Bain C.J., Battistutta D., Ness A.R., Paissat D. and Maclennan R. Betel quid not containing tobacco and oral cancer: A report on a case-control study in Papua New Guinea and a meta-analysis of current evidence. *Int J Cancer* 2007; **120**(6): 1318-1323

481. Thomson P.J. Field change and oral cancer: New evidence for widespread carcinogenesis? *Int J Oral Max Surg* 2002; **31**(3): 262-266

482. Tommasino M., Adamczewski J.P., Carlotti F., Barth C.F., Manetti R., Contorni M., Cavalieri F., Hunt T. and Crawford L. HPV16 E7 protein associates with the protein kinase p33CDK2 and cyclin A. *Oncogene* 1993; **8**(1): 195-202

483. Torres-Rendon A., Stewart R., Craig G.T., Wells M. and Speight P.M. DNA ploidy analysis by image cytometry helps to identify oral epithelial dysplasias with a high risk of malignant progression. *Oral Oncol* 2009; **45**(6): 468-473

484. Tran T.N, Liu Y., Takagi M., Yamaguchi A. and Fujii H. Frequent promoter hypermethylation of RASSF1A and $p16^{INK4a}$ and infrequent allelic loss other than

9p21 in betel-associated oral carcinoma in a Vietnamese non-smoking/non-drinking female population. *J Oral Pathol Med* 2005; **34**(3): 150-156

485. Tsai C.H., Yang C.C., Chou L.S.S. and Chou M.Y. The correlation between alteration of p16 gene and clinical status in oral squamous cell carcinoma. *J Oral Pathol Med* 2001; **30**(9): 527-531

486. Tsai H.-J., Peng Y.-W., Lin L.-Y., Chou M.-C., Lee H. and Chiou H.-L. An association between human papillomavirus 16/18 deoxyribonucleic acid in peripheral blood with p16 protein expression in neoplastic cervical lesions. *Cancer Detect Prev* 2005; **29**(6): 537-543

487. Tsoumpou I., Arbyn M., Kyrgiou M., Wentzensen N., Koliopoulos G., Martin-Hirsch P., Malamou-Mitsi V. and Paraskevaidis E. p16$^{INK4a}$ immunostaining in cytological and histological specimens from the uterine cervix: A systematic review and meta-analysis. *Cancer Treat Rev* 2009; **35**(3): 210-220

488. Tsui I.F.L., Poh C.F., Garnis C., Rosin M.P., Zhang L. and Lam W.L. Multiple pathways in the FGF signaling network are frequently deregulated by gene amplification in oral dysplasias. *Int J Cancer* 2009; **125**(9): 2219-2228

489. Ustav M., Ustav E., Szymanski P. and Stenkindt A. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. *EMBO J* 1991; **10**(13): 4321-4329

490. Uzawa N., Sonoda I., Myo K., Takahashi K-I, Miyamoto R. and Amagasa T. Fluorescence in situ hybridization for detecting genomic alterations of cyclin D1 and p16 in oral squamous cell carcinomas. *Cancer* 2007; **110**(10): 2230-2239

491. Van der Schroeff M.P. and de Jong R.J.B. Staging and prognosis in head and neck cancer. *Oral Oncol* 2009; **45**(4-5): 356-360

492. Van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncol* 2009; **45**(4-5): 317-323

493. Van Doorn L., Molijn A., Kleter B., Quint W. and Colau B. Highly effective detection of human papillomavirus 16 and 18 DNA by a testing algorithm combining broad-spectrum and type-specific PCR. *J Clin Microbiol* 2006; **44**(9): 3292-3298

494. Van Oijen M.G.C.T., Van de Craats J.G. and Slootweg P.J. p53 overexpression in oral mucosa in relation to smoking. *J Pathol* 1999; **187**(4): 469-474

495. Van Oijen M.G.C.T. and Slootweg P.J. Oral field cancerization: Carcinogen-induced independent events or micrometastatic deposits? *Cancer Epidem Biomar* 2000; **9**(3): 249-256

496. Van Rees B.E., Cleton-Jansen A., Cense H.A., Polak M.M., Clement M.J., Drillenburg P., van Lanschot J.J.B. and Offenhaus G.J.A. Molecular evidence of

field cancerization in a patient with 7 tumors of the aerodigestive tract. *Hum Pathol* 2000; **31**(2): 269-271

497. Varshney P.K., Agrawal N. and Bariar L.M. Tobacco and alcohol consumption in relation to oral cancer. *Indian J Otolaryngol Head Neck Surg* 2003; **55**(1): 25-28

498. Villa L.L. Chapter 1: Biology of genital human papillomaviruses. *Int J Gynecol and Obstet* 2006; **94**(S1): S3-S7

499. Vincent R.G. and Marchetta F. The relationship of the use of tobacco and alcohol to cancer of the oral cavity, pharynx or larynx. *Am J Surg* 1963; **106**(3): 501-505

500. Viswanathan M., Tsuchida N. and Shanmugam G. Promoter hypermethylation profile of tumour-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. *Int J Cancer* 2003; **105**(1): 41-46

501. Von Zeidler S.V., Miracca E.C., Nagai M.A. and Birman E.G. Hypermethylation of the p16 gene in normal oral mucosa of smokers. *Int J Mol Med* 2004; **14**(5): 807-811

502. Vora A.R. Alcohol, tobacco and paan use and understanding of oral cancer risk among Asian males in Leicester. *Brit Dent J* 2000; **188**(8): 444-451

503. Vriesema J.L.J., Aben K.K.H., Witjes J.A., Kiemeney L.A.L.M. and Schalken J.A. Superficial and metachronous invasive bladder carcinomas are clonally related. *Int J Cancer* 2001; **93**(5): 699-702

504. Wang A.X., Xu H.S., Lia K.Q. and Zhong X.L. Meta analysis of the relationship between tumorigenesis of oral squamous cell carcinoma and human papillomavirus infection. Ai zheng = Aizheng = *Chin J Cancer Res* 2004; **23**(9): 1077-1080

505. Warnakulasuriya K.A.A.S. and Johnson N.W. Dentists and oral cancer prevention in the UK: Opinions, attitudes and practices to screening for mucosal lesions and to counselling patients on tobacco and alcohol use: Baseline data from 1991. *Oral Dis* 1999; **5**(1): 10-14

506. Warnakulasuriya S., Reibel J., Bouquot J. and Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weakness and scope for improvement. *J Oral Pathol Med* 2008; **37**(3): 127-133

507. Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol* 2009; **45**(4-5): 309-316

508. Weijers M., Snow G.B., Dick Bezemer P. and van der Waal I. Malignancy grading is no better than conventional histopathological grading in small squamous cell carcinoma of tongue and floor of mouth: Retrospective study in 128 patients. *J Oral Pathol Med* 2009; **38**(4): 343-347

509. Weinberger P.M., Yu Z., Haffty B.G., Kowalski D., Harigopal M., Sasaki C., Rimm D.L. and Psyrri A. Prognostic Significance of p16 protein levels in oropharyngeal squamous cell cancer. *Clin Cancer Res* 2004; **10**(17): 5684-5691

510. Weitkunat R., Sanders E. and Lee P.N. Meta-analysis of the relation between European and American smokeless tobacco and oral cancer. *BMC Public Health* 2007; **7**: 334

511. Wentzensen N., Vinokurova S. and Doeberitz M. v. K. Systematic Review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of female lower genital tract. *Cancer Res* 2004; **64**(11): 3878-3884

512. Wey P.D., Lotz M.J. and Triedman L.J. Oral cancer in women nonusers of tobaccco and alcohol. *Cancer* 1987; **60**(7): 1644-1650

513. Wickham C.L., Boyce M., Joyner M.V., Sarsfield P., Wilkins B.S., Jones D.B. and Ellard S. Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *J Clin Pathol Mol Pathol* 2000; **53**(1): 19-23

514. Wight A.J. and Ogden G.R. Possible mechanisms by which alcohol may influence the development of oral cancer - A review. *Oral Oncol* 1998; **34**(6): 441-447

515. Wilson V.G., West M., Woytek K. and Rangasamy D. Papillomavirus E1 proteins: form, function, and features. *Virus Genes* 2002; **24**(3): 275-290

516. Wittekindt C., Gültekin E., Weissenborn S.J., Dienes H.P., Pfister H.J. and Klussmann J.P. Expression of p16 protein is associated with human papillomavirus status in tonsillar carcinomas and has implications on survival. *Adv Oto-Rhino-Laryng* 2005; **62**: 72-80

517. Woolgar J.A. Rogers S.N., Lowe D, Brown J.S. and Vaughan E.D. Cervical lymph node metastasis in oral cancer: the importance of even microscopic extracapsular spread. *Oral Oncol* 2003; **39**(2): 130-137

518. Woolgar J.A. and Triantafyllou A. A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. *Oral Oncol* 2005; **41**(10): 1034-1043

519. Woolgar J.A. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol* 2006; **42**(3): 229-239

520. Woolgar J.A. and Hall G.L. Determinants of outcome following surgery for oral squamous cell carcinoma. *Future Oncol* 2009; **5**(1): 51-61

521. Woolgar J.A. and Triantafyllou, A. Pitfalls and procedures in the histopathological diagnosis of oral and oropharyngeal squamous cell carcinoma and a review of the role of pathology in prognosis. *Oral Oncol* 2009; **45**(4-5): 361-385

522. Worsham M.J., Wolman S.R., Carey T.E., Zarbo R.J., Benninger D.L. and Van Dyke D.L. Common clonal origin of synchronous primary head and neck squamous cell carcinomas: analysis by tumour karyotypes and fluorescence in situ hybridisation. *Hum Pathol* 1995; **26**(3): 251-261

523. Wu L., Patten N., Yamashiro C.T. and Chui B. Extraction and amplification of DNA from formalin-fixed, paraffin-embedded tissues. *Appl Immunohisto M M* 2002; **10**(3): 269-274

524. Yang S.W., Lee Y.S., Chen T.A., Wu C.J. and Tsai C.N. Human papillomavirus in oral leukoplakia in no prognostic indicator of malignant transformation. *Cancer Epidem* 2009; **33**(2): 118-122

525. Yakushiji T., Uzawa K., Shibahara T., Noma H., Tanzawa H. Over-expression of DNA methyltransferases and CDKN2A gene methylation status in squamous cell carcinoma of the oral cavity. *Int J Oncol* 2003; **22**(6): 1201-1207

526. Ying-Chin K., Yung-Li H., Chien-Hung L., Mei-Ju C., Li-Min L. and Chih-Cheng T. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. *J Oral Pathol Med* 1995; **24**(10): 450-453

527. Yokoyama A. and Omori T. Genetic Polymorphisms of Alcohol and Aldehyde Dehydrogenases and Risk for Esophageal and Head and Neck Cancers. *Jpn J Clin Oncol* 2003; **33**(3): 111-121

528. Yoshida T., Sano T., Kanuma T., Owada N., Sakurai S., Fukuda T. and Nakajima T. Immunochemical Analysis of HPV L1 Capsid Protein and p16 Protein in Liquid-based Cytology Samples From Uterine Cervical Lesions. *Cancer* 2008; **114**(2): 83-88

529. Yuen P.W., Man M., Lam K.Y. and Kwong Y.L. Clinicopathological significance of p16 gene expression in the surgical treatment of head and neck squamous cell carcinomas. *J Clin Pathol* 2002; **55**(1): 58-60

530. Zain R.B. Cultural and dietary risk factors of oral cancer and precancer – a brief overview. *Oral Oncol* 2001; **37**(3): 205-210

531. Zarei M.R., Moradie A., Hamkar R., Mohammadalizadeh S., Chamani G., Alizadeh N., Kakooei S. and Eslami B. Detection of human papillomavirus DNA sequences in oral lesions using polymerase chain reaction. *Acta Medica Iranica* 2007; **45**(3): 177-182

532. Zavras A.I., Douglass C.W., Joshipura K., Wu T., Laskaris G., Petridou E., Dokianakis G., Segas J., Lefantzis D., Nomikos P., Wang Y.F. and Diehl, S.R. Smoking and alcohol in the etiology of oral cancer: Gender-specific risk profiles in the south of Greece. *Oral Oncol* 2001; **37**(1): 28-35

533. Zavras A.I., Wu T., Laskaris G., Wang Y.-F., Cartsos V., Segas J., Lefantzis D., Joshipura K., Douglass C.W. and Diehl S.R. Interaction between a single

nucleotide polymorphism in the alcohol dehydrogenase 3 gene, alcohol consumption and oral cancer risk. *Int J Cancer* 2002; **97**(4): 526-530

534. Zhang Z.-Y., Sdek P., Cao J. and Chen W.-T. Human papillomavirus type 16 and 18 DNA in oral squamous cell carcinoma and normal mucosa. *Int J Oral Max Surg* 2004; **33**(1): 71-74

535. Zheng T., Boyle P., Hu H., Duan J., Jiang P., Ma D., Shui L., Niu S. and MacMahon B. Tobacco smoking, alcohol consumption, and risk of oral cancer: a case-control study in Beijing, People's Republic of China. *Cancer Cause Control* 1990; **1**(2): 173-179

536. Znaori A., Brennan P., Gajalakshmi V., Mathew A., Shanta V., Varghese C. and Boffetta P. Independent and combined effects of tobacco smoking, chewing and alcohol drinking on the risk of oral, pharyngeal and esophageal cancers in Indian men. *Int J Cancer* 2003; **105**(5): 681-686

537. Zur Hausen H. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* 1991; **184**(1): 9-13

538. Zur Hausen H. Viruses in human cancers. *Science* 1991; **254**(5035): 1167-1173

539. Zur Hausen H. Papillomavirus infections – a major cause of human cancers. *Biochim Biophys Acta* 1996; **1288**(2): F55-F78

540. http://info.cancerresearchuk.org/cancerstats/types/oral/incidence/

541. http://info.cancerresearchuk.org/cancerstats/types/oral/mortality/

542. http://www.dnabaser.com/, Heracle Software, Germany

543. http://www.dna-software.co.uk/, University of Durham

544. http://www.ebi.ac.uk/Information/ebi_bac/, European Molecular Biology Laboratory

## Appendix 1

### PCR reagents preparation

*Distilled water*:

Distilled water was molecular biology grade, DNase and RNase free, ordered from Sigma (W4502, 1 liter quantity). Distilled water was aliquoted into 20 ml aliquots and stored at ambient temperature.

*Primers*:

Universal primer sets (GH20/PC04, MY11/MY09/GP6 and GP5+/GP6+) and type-specific primer pairs (SPF1/2) for HPV genotype 16, 18, 31 and 33 were ordered in a 200 ηmole quantity from Invitrogen. Each primer pellet was resuspended in sterile distilled water to make 100 μM stock separately. For each primer pairs, 6 μl stock was added to 94 μl sterile distilled water to make 6 μM which is equivalent to 6 ρmole/μl.

*dNTPs*:

dNTP set comprising 500 μl of each dATP, dCTP, dGTP and dTTP at 10 mM concentration were ordered from AB gene (order number AB-0241). 100 μl of each dATP, dCTP, dGTP and dTTP stock were mixed to prepare a dNTP mix with final concentration of 2.5 mM of each nucleotide.

*Amplitaq Gold enzyme:*

Amplitaq Gold 6 comprising 6 x 250 units of Amplitaq Gold at 5U/μl, 10x PCR buffer II and 25 mM $MgCl_2$ solution was ordered from Applied Biosystems (order number N808-0243).

GoTaq® Green Master Mix, 2X (Cat. # M7112, for 100 reactions), Promega Corp.

It was supplied with GoTaq® DNA polymerase, GoTaq® Reaction Buffer (pH 8.5, with yellow and blue loading dyes), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP and 3 mM $MgCl_2$ and Nuclease-Free Water.

# Appendix 2

## Positive Control DNAs

### Isolation of DNA from 3T3 Embryonic Mouse Fibroblast Cells

Two vials containing a pellet of 3T3 embryonic mouse fibroblast cells, each of which contained $0.9 \times 10^6$ (B1) and $1.1 \times 10^6$ (B2) cells was used as a positive control for DNA extraction and each was resuspended with 100 μl of sterile distilled water. DNA extractions were carried out using Qiagen techniques and DNA yield and DNA purity were later determined.

### Isolation of DNA from Human Salivary Glands

Two vials containing pellet of human salivary glands cells, $1.0 \times 10^6$ (A1) and $1.6 \times 10^5$ (A2) cells was used as a positive control for DNA extraction, β-globin and HPV gene detection and each was resuspended with 100 μl of sterile distilled water. DNA extractions were carried out using Qiagen techniques and DNA yield and DNA purity were determined.

### Plasmid DNAs containing HPV genes

Plasmids (λ) containing HPV genomic DNA, HPV6 and HPV8 (courtesy of Dr. Karin Purdie, Queen Mary, University of London) and HPV16 (courtesy of Mr. Maurits de Koning, DDL Laboratory, The Netherlands). Each plasmid was provided at the concentration of 100 ηg/μl and was used as a positive control in HPV gene detection.

# Appendix 3

## Reagents preparation for Fast Blast DNA stain

*100X Fast Blast DNA stain for quick staining (15 min)*

100 ml of 500X Fast Blast DNA was added to 400 ml of deionised water in 1 litre conical flask. The diluted solution was thoroughly mixed and the flask was covered and stored at ambient temperature.

*1X Fast Blast DNA stain for overnight staining (overnight)*

1 ml of 500X Fast Blast was added and gently mixed to 499 ml of deionised water in 1 litre conical flask. Alternatively, 5 ml of 100X Fast Blast was added to 495 ml of deionised water and thoroughly mixed. The flask was covered and stored at room temperature.

*Destaining solution*

A large container with 500-700 ml of clean, warm tap water was used to remove the background staining from Fast Blast in the agarose gel. This destaining procedure only was required for quick staining procedure with 100X Fast Blast DNA.

# Appendix 4

## Human β-globin DNA sequence

```
LOCUS       NG_000007               81706 bp    DNA     linear   PRI 25-JAN-2009
DEFINITION  Homo sapiens beta globin region (HBB@); and hemoglobin, beta (HBB);
            and hemoglobin, delta (HBD); and hemoglobin, epsilon 1 (HBE1); and
            hemoglobin, gamma A (HBG1); and hemoglobin, gamma G (HBG2), on
            chromosome 11.
ACCESSION   NG_000007
VERSION     NG_000007.3  GI:28380636
KEYWORDS    RefSeqGene.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens


>GH20 primer
GAAGAGCCAAGGACAGGTAC (70400 to 70419)
>PC04 primer
GGTGAACGTGGATGAAGTTG (70648 to 70669)
human beta globin gene (70545 to 72150)- shaded area

ORIGIN

...
...

    70021 tttcttttct taccagaagg ttttaatcca aataaggaga agatatgctt agaaccgagg
    70081 tagagttttc atccattctg tcctgtaagt attttgcata ttctggagac gcaggaagag
    70141 atccatctac atatcccaaa gctgaattat ggtagacaaa actcttccac ttttagtgca
    70201 tcaacttctt atttgtgtaa taagaaaatt gggaaaacga tcttcaatat gcttaccaag
    70261 ctgtgattcc aaatattacg taaatacact tgcaaaggag gatgtttttа gtagcaattt
    70321 gtactgatgg tatggggcca agagatatat cttagaggga gggctgaggg tttgaagtcc
                                GH20
    70381 aactcctaag ccagtgccag aagagccaag gacaggtacg gctgtcatca cttagacctc
    70441 accctgtgga gccacaccct agggttggcc aatctactcc caggagcagg gagggcagga
    70501 gccagggctg ggcataaaag tcagggcaga gccatctatt gcttacattt gcttctgaca
    70561 caactgtgtt cactagcaac ctcaaacaga caccatggtg catctgactc ctgaggagaa
    70621 gtctgccgtt actgccctgt ggggcaaggt gaacgtggat gaagttggtg gtgaggccct
                                                    PC04
    70681 gggcaggttg gtatcaaggt tacaagacag gtttaaggag accaatagaa actgggcatg
    70741 tggagacaga gaagactctt gggtttctga taggcactga ctctctctgc ctattggtct
    70801 attttcccac ccttaggctg ctggtggtct acccttggac ccagaggttc tttgagtcct
    70861 ttggggatct gtccactcct gatgctgtta tgggcaaccc taaggtgaag gctcatggca
    70921 agaaagtgct cggtgccttt agtgatggcc tggctcacct ggacaacctc aagggcacct
    70981 ttgccacact gagtgagctg cactgtgaca agctgcacgt ggatcctgag aacttcaggg
    71041 tgagtctatg ggacgcttga tgttttcttt ccccttcttt tctatggtta agttcatgtc
    71101 ataggaaggg gataagtaac agggtacagt ttagaatggg aaacagacga atgattgcat
    71161 cagtgtggaa gtctcaggat cgttttagtt tcttttattt gctgttcata acaattgttt
    71221 tcttttgttt aattcttgct ttcttttttt ttcttctccg caatttttac tattatactt
    71281 aatgccttaa cattgtgtat aacaaaagga aatatctctg agatacatta agtaacttaa
    71341 aaaaaaactt tacacagtct gcctagtaca ttactatttg gaatatatgt gtgcttattt
    71401 gcatattcat aatctcccta ctttattttc ttttattttt aattgataca taatcattat
    71461 acatatttat gggttaaagt gtaatgtttt aatatgtgta cacatattga ccaaatcagg
    71521 gtaattttgc atttgtaatt ttaaaaaatg ctttcttctt ttaatatact tttttgttta
    71581 tcttatttct aatactttcc ctaatctctt tctttcaggg caataatgat acaatgtatc
    71641 atgcctcttt gcaccattct aaagaataac agtgataatt tctgggttaa ggcaatagca
    71701 atatctctgc atataaatat ttctgcatat aaattgtaac tgatgtaaga ggtttcatat
    71761 tgctaatagc agctacaatc cagctaccat tctgctttta ttttatggtt gggataaggc
    71821 tggattattc tgagtccaag ctaggccctt ttgctaatca tgttcatacc tcttatcttc
    71881 ctcccacagc tcctgggcaa cgtgctggtc tgtgtgctgg cccatcactt tggcaaagaa
    71941 ttcaccccac cagtgcaggc tgcctatcag aaagtggtgg ctggtgtggc taatgccctg
    72001 gcccacaagt atcactaagc tcgctttctt gctgtccaat ttctattaaa ggttcctttg
    72061 ttccctaagt ccaactacta aactggggga tattatgaag ggccttgagc atctggattc
    72121 tgcctaataa aaaacattta ttttcattgc aatgatgtat ttaaattatt tctgaatatt
...
...
```

# Appendix 5

Base-nucleic acid codes

The Nomenclature Committee of the International Union of Biochemistry (NC-IUB) has established a standard code to represent uncertain or ambiguous bases in addition to the existing standard four base symbols, A, C, G and T in a nucleic acid from raw DNA sequence data.

| Abbreviation | Base/s | Description |
|---|---|---|
| A | A | Adenine |
| C | C | Cytosine |
| G | G | Guanine |
| T | T | Thymine |
| R | A or G | puRine |
| Y | C or T | pYrimidine |
| M | A or C | aMino |
| K | G or T | Keto |
| S | C or G | Strong interactions 3 hydrogen bonds |
| W | A or T | Weak interactions 2 hydrogen bonds |
| H | A, C or T; not G | H follows G in alphabet |
| B | C, G or T; not A | B follows A in alphabet |
| V | A, C of G; not T (not U) | V follows U in alphabet |
| D | A, G or T; not C | D follows C in alphabet |
| N | A, C, G or T | Any base |

Adapted from NC-IUB (1985)

**Appendix 6**

Table A. Quality and quantity of extracted DNA within positive controls (HSGs)

| ID | Replication | $A_{260}$ | $A_{280}$ | DNA purity ($A_{260}/A_{280}$) | DNA yield (μg/μl) |
|---|---|---|---|---|---|
| A1.1 | 1 | 0.039 | 0.015 | 2.600 | 0.40 |
| A1.1 | 2 | 0.027 | 0.016 | 1.688 | 0.39 |
| A1.1 | 3 | 0.037 | 0.013 | 2.846 | 0.38 |
| A1.2 | 1 | 0.049 | 0.036 | 1.361 | 0.53 |
| A1.2 | 2 | 0.037 | 0.027 | 1.370 | 0.69 |
| A1.2 | 3 | 0.049 | 0.035 | 1.400 | 0.69 |
| A2.1 | 1 | 0.034 | 0.018 | 1.889 | 0.38 |
| A2.1 | 2 | 0.040 | 0.020 | 2.000 | 0.42 |
| A2.1 | 3 | 0.037 | 0.018 | 2.056 | 0.35 |
| A2.2 | 1 | 0.104 | 0.064 | 1.625 | 1.04 |
| A2.2 | 2 | 0.101 | 0.066 | 1.530 | 1.05 |
| A2.2 | 3 | 0.102 | 0.054 | 1.889 | 1.06 |

Note: A1 and A2 – DNA extracted from human salivary glands (HSGs) in duplicate

Table B. Comparison between positive controls (HSGs and 3T3)

| ID | Replication | $A_{260}$ | $A_{280}$ | DNA purity ($A_{260}/A_{280}$) | DNA yield (μg/μl) |
|---|---|---|---|---|---|
| A1 | 1 | 0.037 | 0.017 | 2.244 | 0.39 |
| A1 | 2 | 0.034 | 0.018 | 1.844 | 0.41 |
| A1 | 3 | 0.037 | 0.016 | 2.451 | 0.37 |
| A2 | 1 | 0.077 | 0.050 | 1.493 | 0.79 |
| A2 | 2 | 0.069 | 0.047 | 1.450 | 0.87 |
| A2 | 3 | 0.076 | 0.045 | 1.644 | 0.88 |
| B1 | 1 | 0.074 | 0.050 | 1.480 | 0.68 |
| B1 | 2 | 0.069 | 0.050 | 1.380 | 0.71 |
| B1 | 3 | 0.061 | 0.049 | 1.245 | 0.75 |
| B2 | 1 | 0.015 | 0.003 | 5.000 | 0.28 |
| B2 | 2 | 0.025 | 0.011 | 2.273 | 0.29 |
| B2 | 3 | 0.016 | 0.007 | 2.286 | 0.29 |

Notes:
A1 and A2 – DNA extracted from HSGs (average measurements from Table A)
B1 and B2 – DNA extracted from 3T3 embryonic mouse fibroblasts

## Appendix 7

Table A. Quality and quantity of extracted DNA within randomly selected FFPETs

| ID | Replication | $A_{260}$ | $A_{280}$ | DNA purity ($A_{260}/A_{280}$) | DNA yield (µg/µl) |
|---|---|---|---|---|---|
| C1 | 1 | 0.113 | 0.067 | 1.687 | 0.91 |
| C1 | 2 | 0.109 | 0.074 | 1.473 | 0.90 |
| C1 | 3 | 0.106 | 0.075 | 1.413 | 1.01 |
| C1 | 1 | 0.025 | 0.021 | 1.190 | 0.25 |
| C1 | 2 | 0.025 | 0.021 | 1.190 | 0.25 |
| C1 | 3 | 0.026 | 0.022 | 1.182 | 0.23 |
| C2 | 1 | 0.044 | 0.040 | 1.100 | 0.53 |
| C2 | 2 | 0.042 | 0.041 | 1.024 | 0.54 |
| C2 | 3 | 0.047 | 0.042 | 1.119 | 0.52 |
| C2 | 1 | 0.042 | 0.035 | 1.200 | 0.34 |
| C2 | 2 | 0.036 | 0.035 | 1.029 | 0.35 |
| C2 | 3 | 0.034 | 0.034 | 1.000 | 0.42 |

Note: C1 and C2 – DNA extracted from selected FFPETs in duplicate

Table B. Comparison between randomly selected 6 FFPETs

| ID | Replication | $A_{260}$ | $A_{280}$ | DNA purity ($A_{260}/A_{280}$) | DNA yield (µg/µl) |
|---|---|---|---|---|---|
| C1 | 1 | 0.069 | 0.044 | 1.439 | 0.58 |
| C1 | 2 | 0.067 | 0.048 | 1.332 | 0.58 |
| C1 | 3 | 0.066 | 0.049 | 1.298 | 0.62 |
| C2 | 1 | 0.035 | 0.031 | 1.145 | 0.39 |
| C2 | 2 | 0.034 | 0.031 | 1.107 | 0.40 |
| C2 | 3 | 0.037 | 0.032 | 1.150 | 0.38 |
| C3 | 1 | 0.027 | 0.022 | 1.227 | 0.27 |
| C3 | 2 | 0.026 | 0.018 | 1.444 | 0.25 |
| C3 | 3 | 0.020 | 0.013 | 1.538 | 0.28 |
| C4 | 1 | 0.019 | 0.020 | 0.950 | 0.01 |
| C4 | 2 | 0.020 | 0.017 | 1.176 | 0.11 |
| C4 | 3 | 0.017 | 0.018 | 0.944 | 0.08 |
| C5 | 1 | 0.027 | 0.025 | 1.080 | 0.20 |
| C5 | 2 | 0.025 | 0.024 | 1.042 | 0.26 |
| C5 | 3 | 0.029 | 0.024 | 1.208 | 0.24 |
| C6 | 1 | 0.004 | 0.001 | 4.000 | 0.04 |
| C6 | 2 | 0.007 | 0.002 | 3.500 | 0.04 |
| C6 | 3 | 0.009 | 0.015 | 0.600 | 0.05 |

Notes:
C1 and C2 – DNA extracted from FFPETs (average measurements from Table A)
C3 to C6 –DNA extracted from randomly selected FFPETs

## Appendix 8

DNA quantification of selected five FFPETs using Qiagen kit (QIAamp DNA FFPE)

| | Sections | | | Reading 1 | | | Reading 2 | | | Reading 3 | | | DNA purity | DNA yield |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | (5 μm) | Tissue | Replication | A260 | A280 | Ratio | A260 | A280 | Ratio | A260 | A280 | Ratio | (Average) | (μg/μl) |
| 1 | 3 | D1 | 1 | 0.458 | 0.339 | 1.351 | 0.462 | 0.338 | 1.367 | 0.459 | 0.338 | 1.358 | 1.359 | 4.597 |
| 2 | | | 2 | 0.471 | 0.345 | 1.365 | 0.471 | 0.345 | 1.365 | 0.471 | 0.343 | 1.373 | 1.368 | 4.710 |
| 3 | | | 3 | 0.448 | 0.327 | 1.370 | 0.448 | 0.326 | 1.374 | 0.446 | 0.323 | 1.381 | 1.375 | 4.470 |
| 4 | | D2 | 1 | 0.461 | 0.337 | 1.368 | 0.462 | 0.339 | 1.363 | 0.461 | 0.338 | 1.364 | 1.365 | 4.617 |
| 5 | | | 2 | 0.469 | 0.341 | 1.375 | 0.473 | 0.344 | 1.375 | 0.472 | 0.344 | 1.372 | 1.374 | 4.723 |
| 6 | | | 3 | 0.450 | 0.326 | 1.380 | 0.449 | 0.328 | 1.369 | 0.450 | 0.330 | 1.364 | 1.371 | 4.487 |
| 7 | | D3 | 1 | 0.454 | 0.338 | 1.343 | 0.459 | 0.339 | 1.354 | 0.457 | 0.342 | 1.336 | 1.344 | 4.560 |
| 8 | | | 2 | 0.466 | 0.343 | 1.359 | 0.467 | 0.345 | 1.354 | 0.470 | 0.347 | 1.354 | 1.356 | 4.670 |
| 9 | | | 3 | 0.443 | 0.326 | 1.359 | 0.442 | 0.326 | 1.356 | 0.443 | 0.326 | 1.359 | 1.358 | 4.447 |
| 10 | | D4 | 1 | 0.444 | 0.339 | 1.310 | 0.445 | 0.339 | 1.313 | 0.451 | 0.345 | 1.307 | 1.310 | 4.447 |
| 11 | | | 2 | 0.464 | 0.345 | 1.345 | 0.451 | 0.345 | 1.307 | 0.454 | 0.343 | 1.324 | 1.325 | 4.543 |
| 12 | | | 3 | 0.427 | 0.326 | 1.310 | 0.430 | 0.324 | 1.327 | 0.428 | 0.321 | 1.333 | 1.323 | 4.280 |
| 13 | | D5 | 1 | 0.457 | 0.337 | 1.356 | 0.459 | 0.340 | 1.350 | 0.460 | 0.337 | 1.365 | 1.357 | 4.617 |
| 14 | | | 2 | 0.468 | 0.344 | 1.360 | 0.470 | 0.343 | 1.370 | 0.469 | 0.344 | 1.363 | 1.365 | 4.677 |
| 15 | | | 3 | 0.441 | 0.325 | 1.357 | 0.442 | 0.323 | 1.368 | 0.441 | 0.322 | 1.370 | 1.365 | 4.393 |
| 16 | 5 | D1 | 1 | 0.456 | 0.341 | 1.337 | 0.456 | 0.338 | 1.349 | 0.456 | 0.339 | 1.345 | 1.344 | 4.560 |
| 17 | | | 2 | 0.467 | 0.357 | 1.308 | 0.467 | 0.353 | 1.323 | 0.467 | 0.346 | 1.350 | 1.327 | 4.670 |
| 18 | | | 3 | 0.441 | 0.326 | 1.353 | 0.445 | 0.327 | 1.361 | 0.444 | 0.327 | 1.358 | 1.357 | 4.493 |
| 19 | | D2 | 1 | 0.458 | 0.332 | 1.380 | 0.460 | 0.334 | 1.377 | 0.459 | 0.335 | 1.370 | 1.376 | 4.580 |
| 20 | | | 2 | 0.468 | 0.341 | 1.372 | 0.467 | 0.335 | 1.394 | 0.467 | 0.339 | 1.378 | 1.381 | 4.667 |
| 21 | | | 3 | 0.476 | 0.350 | 1.360 | 0.477 | 0.350 | 1.363 | 0.443 | 0.328 | 1.351 | 1.358 | 4.643 |
| 22 | | D3 | 1 | 0.453 | 0.333 | 1.360 | 0.454 | 0.331 | 1.372 | 0.453 | 0.334 | 1.356 | 1.363 | 4.537 |
| 23 | | | 2 | 0.460 | 0.344 | 1.337 | 0.464 | 0.339 | 1.369 | 0.464 | 0.337 | 1.377 | 1.361 | 4.637 |
| 24 | | | 3 | 0.443 | 0.320 | 1.384 | 0.443 | 0.329 | 1.347 | 0.443 | 0.321 | 1.380 | 1.370 | 4.413 |
| 25 | | D4 | 1 | 0.454 | 0.331 | 1.372 | 0.457 | 0.330 | 1.385 | 0.457 | 0.330 | 1.385 | 1.380 | 4.550 |
| 26 | | | 2 | 0.465 | 0.338 | 1.376 | 0.465 | 0.338 | 1.376 | 0.468 | 0.337 | 1.389 | 1.380 | 4.647 |
| 27 | | | 3 | 0.443 | 0.321 | 1.380 | 0.442 | 0.322 | 1.373 | 0.442 | 0.324 | 1.364 | 1.372 | 4.443 |
| 28 | | D5 | 1 | 0.458 | 0.335 | 1.367 | 0.457 | 0.338 | 1.352 | 0.457 | 0.334 | 1.368 | 1.362 | 4.593 |
| 29 | | | 2 | 0.464 | 0.338 | 1.373 | 0.469 | 0.338 | 1.388 | 0.467 | 0.337 | 1.386 | 1.382 | 4.687 |
| 30 | | | 3 | 0.441 | 0.322 | 1.370 | 0.445 | 0.319 | 1.395 | 0.445 | 0.319 | 1.395 | 1.387 | 4.457 |
| 31 | 10 | D1 | 1 | 0.447 | 0.330 | 1.355 | 0.448 | 0.333 | 1.345 | 0.449 | 0.335 | 1.340 | 1.347 | 4.503 |
| 32 | | | 2 | 0.456 | 0.337 | 1.353 | 0.455 | 0.335 | 1.358 | 0.456 | 0.335 | 1.361 | 1.358 | 4.557 |
| 33 | | | 3 | 0.436 | 0.321 | 1.358 | 0.435 | 0.320 | 1.359 | 0.439 | 0.322 | 1.363 | 1.360 | 4.390 |
| 34 | | D2 | 1 | 0.455 | 0.334 | 1.362 | 0.454 | 0.333 | 1.363 | 0.455 | 0.334 | 1.362 | 1.363 | 4.550 |
| 35 | | | 2 | 0.459 | 0.338 | 1.358 | 0.462 | 0.335 | 1.379 | 0.466 | 0.336 | 1.387 | 1.375 | 4.640 |
| 36 | | | 3 | 0.443 | 0.320 | 1.384 | 0.443 | 0.320 | 1.384 | 0.443 | 0.316 | 1.402 | 1.390 | 4.450 |
| 37 | | D3 | 1 | 0.461 | 0.337 | 1.368 | 0.463 | 0.339 | 1.366 | 0.466 | 0.336 | 1.387 | 1.374 | 4.627 |
| 38 | | | 2 | 0.471 | 0.340 | 1.385 | 0.469 | 0.341 | 1.375 | 0.472 | 0.347 | 1.360 | 1.374 | 4.730 |
| 39 | | | 3 | 0.446 | 0.325 | 1.372 | 0.449 | 0.327 | 1.373 | 0.452 | 0.327 | 1.382 | 1.376 | 4.490 |
| 40 | | D4 | 1 | 0.452 | 0.332 | 1.361 | 0.464 | 0.335 | 1.385 | 0.456 | 0.334 | 1.365 | 1.371 | 4.563 |
| 41 | | | 2 | 0.467 | 0.344 | 1.358 | 0.465 | 0.338 | 1.376 | 0.469 | 0.342 | 1.371 | 1.368 | 4.670 |
| 42 | | | 3 | 0.443 | 0.322 | 1.376 | 0.448 | 0.325 | 1.378 | 0.447 | 0.323 | 1.384 | 1.379 | 4.450 |
| 43 | | D5 | 1 | 0.455 | 0.335 | 1.358 | 0.460 | 0.333 | 1.381 | 0.457 | 0.330 | 1.385 | 1.375 | 4.573 |
| 44 | | | 2 | 0.462 | 0.342 | 1.351 | 0.466 | 0.335 | 1.391 | 0.465 | 0.339 | 1.372 | 1.371 | 4.653 |
| 45 | | | 3 | 0.449 | 0.324 | 1.386 | 0.446 | 0.325 | 1.372 | 0.453 | 0.326 | 1.390 | 1.383 | 4.463 |

## Appendix 9

## One-way ANOVA on extracted DNA using Qiagen kit

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| DNA purity | 3 coupes | 15 | 1.35433 | 0.019963 | 0.005154 | 1.34328 | 1.36539 | 1.310 | 1.375 |
| | 5 coupes | 15 | 1.36667 | 0.016167 | 0.004174 | 1.35771 | 1.37562 | 1.327 | 1.387 |
| | 10 coupes | 15 | 1.37093 | 0.010633 | 0.002746 | 1.36504 | 1.37682 | 1.347 | 1.390 |
| | Total | 45 | 1.36398 | 0.017222 | 0.002567 | 1.35880 | 1.36915 | 1.310 | 1.390 |
| DNA concentration | 3 coupes | 15 | 4.54920 | 0.127201 | 0.032843 | 4.47876 | 4.61964 | 4.280 | 4.723 |
| | 5 coupes | 15 | 4.57180 | 0.088838 | 0.022938 | 4.52260 | 4.62100 | 4.413 | 4.687 |
| | 10 coupes | 15 | 4.55393 | 0.096707 | 0.024970 | 4.50038 | 4.60749 | 4.390 | 4.730 |
| | Total | 45 | 4.55831 | 0.103595 | 0.015443 | 4.52719 | 4.58943 | 4.280 | 4.730 |

**Test of Homogeneity of Variances**

| | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|
| DNA purity | 2.322 | 2 | 42 | 0.111 |
| DNA concentration | 1.252 | 2 | 42 | 0.296 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| DNA purity | Between Groups | 0.002 | 2 | 0.001 | 4.326 | 0.020 |
| | Within Groups | 0.011 | 42 | 0.000 | | |
| | Total | 0.013 | 44 | | | |
| DNA concentration | Between Groups | 0.004 | 2 | 0.002 | 0.191 | 0.827 |
| | Within Groups | 0.468 | 42 | 0.011 | | |
| | Total | 0.472 | 44 | | | |

## Appendix 9 (contd.)

**Post Hoc Test: Multiple Comparisons**

| Dependent Variable | | (I) Coupe number | (J) Coupe number | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Lower Bound | Upper Bound |
| DNA purity | Tukey HSD | 3 coupes | 5 coupes | -0.012333 | 0.005861 | 0.101 | -0.02657 | 0.00191 |
| | | | 10 coupes | -0.016600* | 0.005861 | 0.019 | -0.03084 | -0.00236 |
| | | 5 coupes | 3 coupes | 0.012333 | 0.005861 | 0.101 | -0.00191 | 0.02657 |
| | | | 10 coupes | -0.004267 | 0.005861 | 0.748 | -0.01851 | 0.00997 |
| | | 10 coupes | 3 coupes | 0.016600* | 0.005861 | 0.019 | 0.00236 | 0.03084 |
| | | | 5 coupes | 0.004267 | 0.005861 | 0.748 | -0.00997 | 0.01851 |
| | Games-Howell | 3 coupes | 5 coupes | -0.012333 | 0.006633 | 0.170 | -0.02878 | 0.00412 |
| | | | 10 coupes | -0.016600* | 0.005840 | 0.025 | -0.03130 | -0.00190 |
| | | 5 coupes | 3 coupes | 0.012333 | 0.006633 | 0.170 | -0.00412 | 0.02878 |
| | | | 10 coupes | -0.004267 | 0.004996 | 0.674 | -0.01674 | 0.00820 |
| | | 10 coupes | 3 coupes | 0.016600* | 0.005840 | 0.025 | 0.00190 | 0.03130 |
| | | | 5 coupes | 0.004267 | 0.004996 | 0.674 | -0.00820 | 0.01674 |
| DNA concentration | Tukey HSD | 3 coupes | 5 coupes | -0.022600 | 0.038543 | 0.828 | -0.11624 | 0.07104 |
| | | | 10 coupes | -0.004733 | 0.038543 | 0.992 | -0.09837 | 0.08891 |
| | | 5 coupes | 3 coupes | 0.022600 | 0.038543 | 0.828 | -0.07104 | 0.11624 |
| | | | 10 coupes | 0.017867 | 0.038543 | 0.889 | -0.07577 | 0.11151 |
| | | 10 coupes | 3 coupes | 0.004733 | 0.038543 | 0.992 | -0.08891 | 0.09837 |
| | | | 5 coupes | -0.017867 | 0.038543 | 0.889 | -0.11151 | 0.07577 |
| | Games-Howell | 3 coupes | 5 coupes | -0.022600 | 0.040060 | 0.840 | -0.12237 | 0.07717 |
| | | | 10 coupes | -0.004733 | 0.041257 | 0.993 | -0.10722 | 0.09776 |
| | | 5 coupes | 3 coupes | 0.022600 | 0.040060 | 0.840 | -0.07717 | 0.12237 |
| | | | 10 coupes | 0.017867 | 0.033906 | 0.859 | -0.06606 | 0.10180 |
| | | 10 coupes | 3 coupes | 0.004733 | 0.041257 | 0.993 | -0.09776 | 0.10722 |
| | | | 5 coupes | -0.017867 | 0.033906 | 0.859 | -0.10180 | 0.06606 |

*. The mean difference is significant at the 0.05 level.

# Appendix 10

## DNA quantification of selected five FFPETs using PROMEGA kit (Magnesil Genomic)

| No. | Sections (5 µm) | Tissue | Replication | Reading 1 | | | Reading 2 | | | Reading 3 | | | DNA purity (average) | DNAyield (µg/µl) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | A260 | A280 | Ratio | A260 | A280 | Ratio | A260 | A280 | Ratio | | |
| 1 | 3 | D1 | 1 | 0.437 | 0.316 | 1.383 | 0.431 | 0.314 | 1.373 | 0.433 | 0.312 | 1.388 | 1.381 | 4.320 |
| 2 | | | 2 | 0.433 | 0.315 | 1.375 | 0.435 | 0.313 | 1.390 | 0.430 | 0.317 | 1.356 | 1.374 | 4.323 |
| 3 | | | 3 | 0.401 | 0.289 | 1.388 | 0.400 | 0.290 | 1.379 | 0.398 | 0.287 | 1.387 | 1.385 | 3.997 |
| 4 | | D2 | 1 | 0.434 | 0.313 | 1.387 | 0.434 | 0.314 | 1.382 | 0.432 | 0.313 | 1.380 | 1.383 | 4.327 |
| 5 | | | 2 | 0.439 | 0.318 | 1.381 | 0.442 | 0.322 | 1.373 | 0.441 | 0.318 | 1.387 | 1.380 | 4.413 |
| 6 | | | 3 | 0.421 | 0.302 | 1.394 | 0.421 | 0.302 | 1.394 | 0.423 | 0.302 | 1.401 | 1.396 | 4.220 |
| 7 | | D3 | 1 | 0.428 | 0.310 | 1.381 | 0.426 | 0.312 | 1.365 | 0.432 | 0.310 | 1.394 | 1.380 | 4.290 |
| 8 | | | 2 | 0.446 | 0.326 | 1.368 | 0.446 | 0.324 | 1.377 | 0.446 | 0.326 | 1.368 | 1.371 | 4.463 |
| 9 | | | 3 | 0.424 | 0.305 | 1.390 | 0.424 | 0.306 | 1.386 | 0.427 | 0.308 | 1.386 | 1.387 | 4.247 |
| 10 | | D4 | 1 | 0.455 | 0.340 | 1.338 | 0.455 | 0.331 | 1.375 | 0.457 | 0.332 | 1.377 | 1.363 | 4.557 |
| 11 | | | 2 | 0.467 | 0.344 | 1.358 | 0.467 | 0.336 | 1.390 | 0.466 | 0.339 | 1.375 | 1.374 | 4.663 |
| 12 | | | 3 | 0.427 | 0.305 | 1.400 | 0.428 | 0.309 | 1.385 | 0.428 | 0.309 | 1.385 | 1.390 | 4.280 |
| 13 | | D5 | 1 | 0.426 | 0.318 | 1.340 | 0.422 | 0.322 | 1.311 | 0.423 | 0.316 | 1.339 | 1.330 | 4.223 |
| 14 | | | 2 | 0.445 | 0.332 | 1.340 | 0.444 | 0.334 | 1.329 | 0.444 | 0.333 | 1.333 | 1.334 | 4.433 |
| 15 | | | 3 | 0.430 | 0.326 | 1.319 | 0.432 | 0.324 | 1.333 | 0.430 | 0.320 | 1.344 | 1.332 | 4.313 |
| 16 | 5 | D1 | 1 | 0.429 | 0.314 | 1.366 | 0.430 | 0.311 | 1.383 | 0.429 | 0.313 | 1.371 | 1.373 | 4.290 |
| 17 | | | 2 | 0.459 | 0.341 | 1.346 | 0.462 | 0.345 | 1.339 | 0.462 | 0.337 | 1.371 | 1.352 | 4.620 |
| 18 | | | 3 | 0.434 | 0.317 | 1.369 | 0.437 | 0.323 | 1.353 | 0.433 | 0.316 | 1.370 | 1.364 | 4.370 |
| 19 | | D2 | 1 | 0.449 | 0.341 | 1.317 | 0.451 | 0.347 | 1.300 | 0.464 | 0.343 | 1.353 | 1.323 | 4.483 |
| 20 | | | 2 | 0.452 | 0.331 | 1.366 | 0.455 | 0.331 | 1.375 | 0.452 | 0.339 | 1.333 | 1.358 | 4.540 |
| 21 | | | 3 | 0.425 | 0.313 | 1.358 | 0.425 | 0.313 | 1.358 | 0.429 | 0.312 | 1.375 | 1.364 | 4.310 |
| 22 | | D3 | 1 | 0.428 | 0.327 | 1.309 | 0.429 | 0.323 | 1.328 | 0.429 | 0.321 | 1.336 | 1.324 | 4.300 |
| 23 | | | 2 | 0.460 | 0.340 | 1.353 | 0.466 | 0.338 | 1.379 | 0.455 | 0.341 | 1.334 | 1.355 | 4.553 |
| 24 | | | 3 | 0.432 | 0.325 | 1.329 | 0.433 | 0.323 | 1.341 | 0.435 | 0.326 | 1.334 | 1.335 | 4.343 |
| 25 | | D4 | 1 | 0.446 | 0.328 | 1.360 | 0.448 | 0.327 | 1.370 | 0.451 | 0.328 | 1.375 | 1.368 | 4.457 |
| 26 | | | 2 | 0.455 | 0.338 | 1.346 | 0.460 | 0.338 | 1.361 | 0.459 | 0.340 | 1.350 | 1.352 | 4.587 |
| 27 | | | 3 | 0.430 | 0.312 | 1.378 | 0.429 | 0.313 | 1.371 | 0.430 | 0.312 | 1.378 | 1.376 | 4.297 |
| 28 | | D5 | 1 | 0.433 | 0.317 | 1.366 | 0.435 | 0.318 | 1.368 | 0.436 | 0.315 | 1.384 | 1.373 | 4.343 |
| 29 | | | 2 | 0.434 | 0.319 | 1.361 | 0.437 | 0.317 | 1.379 | 0.439 | 0.314 | 1.398 | 1.379 | 4.360 |
| 30 | | | 3 | 0.431 | 0.314 | 1.373 | 0.430 | 0.312 | 1.378 | 0.430 | 0.309 | 1.392 | 1.381 | 4.290 |
| 31 | 10 | D1 | 1 | 0.448 | 0.330 | 1.358 | 0.452 | 0.328 | 1.378 | 0.448 | 0.331 | 1.353 | 1.363 | 4.483 |
| 32 | | | 2 | 0.455 | 0.333 | 1.366 | 0.456 | 0.329 | 1.386 | 0.456 | 0.332 | 1.373 | 1.375 | 4.567 |
| 33 | | | 3 | 0.426 | 0.306 | 1.392 | 0.424 | 0.308 | 1.377 | 0.427 | 0.310 | 1.377 | 1.382 | 4.273 |
| 34 | | D2 | 1 | 0.450 | 0.329 | 1.368 | 0.448 | 0.333 | 1.345 | 0.449 | 0.330 | 1.361 | 1.358 | 4.483 |
| 35 | | | 2 | 0.446 | 0.325 | 1.372 | 0.445 | 0.324 | 1.373 | 0.443 | 0.322 | 1.376 | 1.374 | 4.440 |
| 36 | | | 3 | 0.423 | 0.308 | 1.373 | 0.419 | 0.307 | 1.365 | 0.419 | 0.305 | 1.374 | 1.371 | 4.217 |
| 37 | | D3 | 1 | 0.417 | 0.313 | 1.332 | 0.416 | 0.317 | 1.312 | 0.417 | 0.312 | 1.337 | 1.327 | 4.160 |
| 38 | | | 2 | 0.450 | 0.327 | 1.376 | 0.438 | 0.326 | 1.344 | 0.436 | 0.327 | 1.333 | 1.351 | 4.360 |
| 39 | | | 3 | 0.414 | 0.310 | 1.335 | 0.412 | 0.308 | 1.338 | 0.411 | 0.315 | 1.305 | 1.326 | 4.113 |
| 40 | | D4 | 1 | 0.438 | 0.328 | 1.335 | 0.439 | 0.338 | 1.299 | 0.438 | 0.327 | 1.339 | 1.325 | 4.390 |
| 41 | | | 2 | 0.445 | 0.332 | 1.340 | 0.451 | 0.335 | 1.346 | 0.446 | 0.336 | 1.327 | 1.338 | 4.460 |
| 42 | | | 3 | 0.404 | 0.293 | 1.379 | 0.404 | 0.294 | 1.374 | 0.405 | 0.295 | 1.373 | 1.375 | 4.050 |
| 43 | | D5 | 1 | 0.453 | 0.329 | 1.377 | 0.453 | 0.332 | 1.364 | 0.453 | 0.332 | 1.364 | 1.369 | 4.540 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | | | 2 | 0.456 | 0.335 | 1.361 | 0.458 | 0.335 | 1.367 | 0.458 | 0.334 | 1.371 | 1.367 | 4.580 |
| 45 | | | 3 | 0.437 | 0.318 | 1.374 | 0.438 | 0.321 | 1.364 | 0.438 | 0.318 | 1.377 | 1.372 | 4.373 |

## Appendix 11

## One-way ANOVA on extracted DNA using Promega kit

**Descriptives**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| DNA purity | 3 coupes | 15 | 1.37067 | 0.021540 | 0.005561 | 1.35874 | 1.38259 | 1.330 | 1.396 |
| | 5 coupes | 15 | 1.35847 | 0.018715 | 0.004832 | 1.34810 | 1.36883 | 1.323 | 1.381 |
| | 10 coupes | 15 | 1.35820 | 0.019865 | 0.005129 | 1.34720 | 1.36920 | 1.325 | 1.382 |
| | Total | 45 | 1.36244 | 0.020475 | 0.003052 | 1.35629 | 1.36860 | 1.323 | 1.396 |
| DNA concentration | 3 coupes | 15 | 4.33793 | 0.156419 | 0.040387 | 4.25131 | 4.42456 | 3.997 | 4.663 |
| | 5 coupes | 15 | 4.40953 | 0.118641 | 0.030633 | 4.34383 | 4.47523 | 4.290 | 4.620 |
| | 10 coupes | 15 | 4.36593 | 0.168178 | 0.043423 | 4.27280 | 4.45907 | 4.050 | 4.580 |
| | Total | 45 | 4.37113 | 0.148831 | 0.022186 | 4.32642 | 4.41585 | 3.997 | 4.663 |

**Test of Homogeneity of Variances**

| | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|
| DNA purity | 0.127 | 2 | 42 | 0.881 |
| DNA concentration | 0.576 | 2 | 42 | 0.566 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| DNA purity | Between Groups | 0.002 | 2 | 0.001 | 1.888 | 0.164 |
| | Within Groups | 0.017 | 42 | 0.000 | | |
| | Total | 0.018 | 44 | | | |
| DNA concentration | Between Groups | 0.039 | 2 | 0.020 | .877 | 0.424 |
| | Within Groups | 0.936 | 42 | 0.022 | | |
| | Total | 0.975 | 44 | | | |

**Appendix 12**

DNA qualification and quantification comparison between
QIAGEN kit (QIAamp DNA FFPE) and PROMEGA kit (Magnesil Genomic)

| No. | Sections (5 μm) | Tissue | DNA Kits[1] | Average purity | Average DNA concentration (μg/μl) |
|---|---|---|---|---|---|
| 1 | 3 | D1 | P | 1.380 | 4.213 |
| 2 | | | Q | 1.367 | 4.592 |
| 3 | | D2 | P | 1.386 | 4.320 |
| 4 | | | Q | 1.370 | 4.609 |
| 5 | | D3 | P | 1.379 | 4.333 |
| 6 | | | Q | 1.353 | 4.559 |
| 7 | | D4 | P | 1.376 | 4.500 |
| 8 | | | Q | 1.320 | 4.423 |
| 9 | | D5 | P | 1.332 | 4.323 |
| 10 | | | Q | 1.362 | 4.562 |
| 11 | 5 | D1 | P | 1.363 | 4.427 |
| 12 | | | Q | 1.343 | 4.574 |
| 13 | | D2 | P | 1.348 | 4.444 |
| 14 | | | Q | 1.372 | 4.630 |
| 15 | | D3 | P | 1.338 | 4.399 |
| 16 | | | Q | 1.365 | 4.529 |
| 17 | | D4 | P | 1.365 | 4.447 |
| 18 | | | Q | 1.378 | 4.547 |
| 19 | | D5 | P | 1.378 | 4.331 |
| 20 | | | Q | 1.377 | 4.579 |
| 21 | 10 | D1 | P | 1.373 | 4.441 |
| 22 | | | Q | 1.355 | 4.483 |
| 23 | | D2 | P | 1.367 | 4.380 |
| 24 | | | Q | 1.376 | 4.547 |
| 25 | | D3 | P | 1.335 | 4.211 |
| 26 | | | Q | 1.374 | 4.616 |
| 27 | | D4 | P | 1.346 | 4.300 |
| 28 | | | Q | 1.373 | 4.561 |
| 29 | | D5 | P | 1.369 | 4.498 |
| 30 | | | Q | 1.376 | 4.563 |

Note:
[1] P for Promega, DNA Extraction Kits (Magnesil Genomic) and
Q for Qiagen, DNA Extraction Kits (QIAamp DNA FFPE)

# Appendix 13

## Quantification of 60 extracted DNA (QIAamp FFPE DNA kit)

| Sample no. | Pathology ID | Reading 1 | | | Reading 2 | | | Reading 3 | | | Average purity | DNA conc. (µg/µl) | Dilution for PCR 10 ηg/µl (µl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A260 | A280 | Ratio | A260 | A280 | Ratio | A260 | A280 | Ratio | | | DNA | $dH_2O$ | Total |
| 1 | 01209199 2 | 0.036 | 0.040 | 0.900 | 0.049 | 0.050 | 0.980 | 0.040 | 0.040 | 1.000 | 0.960 | 0.477 | 4 | 196 | 200 |
| 2 | 954593 B | 0.044 | 0.035 | 1.257 | 0.047 | 0.045 | 1.044 | 0.047 | 0.045 | 1.044 | 1.115 | 0.463 | 4 | 196 | 200 |
| 3 | NS00196904 1 | 0.062 | 0.061 | 1.016 | 0.068 | 0.065 | 1.046 | 0.063 | 0.061 | 1.033 | 1.032 | 0.603 | 3 | 197 | 200 |
| 4 | NS00442204 1L | 0.059 | 0.040 | 1.475 | 0.047 | 0.033 | 1.424 | 0.057 | 0.043 | 1.326 | 1.408 | 0.647 | 3 | 197 | 200 |
| 5 | NS00642004 1 | 0.050 | 0.040 | 1.250 | 0.048 | 0.045 | 1.067 | 0.050 | 0.046 | 1.087 | 1.135 | 0.497 | 4 | 196 | 200 |
| 6 | NS00671203 1 | 0.047 | 0.040 | 1.175 | 0.049 | 0.033 | 1.485 | 0.041 | 0.038 | 1.079 | 1.246 | 0.510 | 4 | 196 | 200 |
| 7 | NS00711302 1B | 0.018 | 0.013 | 1.385 | 0.017 | 0.008 | 2.125 | 0.020 | 0.015 | 1.333 | 1.614 | 0.200 | 10 | 190 | 200 |
| 8 | NS00848002 3D | 0.065 | 0.063 | 1.032 | 0.067 | 0.062 | 1.081 | 0.067 | 0.066 | 1.015 | 1.043 | 0.680 | 3 | 197 | 200 |
| 9 | NS01/68103 1A | 0.052 | 0.047 | 1.106 | 0.054 | 0.048 | 1.125 | 0.054 | 0.051 | 1.059 | 1.097 | 0.543 | 4 | 196 | 200 |
| 10 | NS01/68103 1B | 0.059 | 0.058 | 1.017 | 0.058 | 0.060 | 0.967 | 0.054 | 0.056 | 0.964 | 0.983 | 0.557 | 4 | 196 | 200 |
| 11 | NS01020203 3K | 0.054 | 0.049 | 1.102 | 0.049 | 0.041 | 1.195 | 0.037 | 0.047 | 0.787 | 1.028 | 0.340 | 6 | 194 | 200 |
| 12 | NS01029003 1 | 0.064 | 0.061 | 1.049 | 0.061 | 0.065 | 0.938 | 0.063 | 0.065 | 0.969 | 0.986 | 0.653 | 3 | 197 | 200 |
| 13 | NS01076404 1B | 0.009 | 0.019 | 0.474 | 0.005 | 0.001 | 5.000 | 0.004 | 0.007 | 0.571 | 2.015 | 0.063 | 32 | 168 | 200 |
| 14 | NS01258903 1C | 0.079 | 0.090 | 0.878 | 0.088 | 0.085 | 1.035 | 0.089 | 0.085 | 1.047 | 0.987 | 0.927 | 2 | 198 | 200 |
| 15 | NS01291204 1C | 0.066 | 0.058 | 1.138 | 0.065 | 0.055 | 1.182 | 0.068 | 0.057 | 1.193 | 1.171 | 0.680 | 3 | 197 | 200 |
| 16 | NS01404702 1B | 0.015 | 0.007 | 2.143 | 0.014 | 0.010 | 1.400 | 0.014 | 0.015 | 0.933 | 1.492 | 0.153 | 13 | 187 | 200 |
| 17 | NS01643002 1A | 0.066 | 0.058 | 1.138 | 0.067 | 0.060 | 1.117 | 0.067 | 0.058 | 1.155 | 1.137 | 0.667 | 3 | 197 | 200 |
| 18 | NS01803103 1 | 0.039 | 0.033 | 1.182 | 0.037 | 0.036 | 1.028 | 0.039 | 0.031 | 1.258 | 1.156 | 0.370 | 5 | 195 | 200 |
| 19 | NS01864704 1B | 0.017 | 0.004 | 4.250 | 0.006 | 0.010 | 0.600 | 0.013 | 0.003 | 4.333 | 3.061 | 0.147 | 14 | 186 | 200 |
| 20 | NS01960104 1 | 0.032 | 0.048 | 0.667 | 0.021 | 0.047 | 0.447 | 0.036 | 0.055 | 0.655 | 0.589 | 0.567 | 4 | 196 | 200 |
| 21 | NS02060804 1 | 0.065 | 0.058 | 1.121 | 0.057 | 0.049 | 1.163 | 0.050 | 0.054 | 0.926 | 1.070 | 0.650 | 3 | 197 | 200 |
| 22 | TS00012706 1 | 0.003 | 0.017 | 0.176 | 0.016 | 0.026 | 0.615 | 0.001 | 0.020 | 0.050 | 0.281 | 0.273 | 7 | 193 | 200 |
| 23 | TS00044505 1 | 0.074 | 0.082 | 0.902 | 0.062 | 0.081 | 0.765 | 0.086 | 0.080 | 1.075 | 0.914 | 0.850 | 2 | 198 | 200 |
| 24 | TS00055407 1 | 0.053 | 0.033 | 1.606 | 0.049 | 0.042 | 1.167 | 0.052 | 0.044 | 1.182 | 1.318 | 0.490 | 4 | 196 | 200 |
| 25 | TS00059407 1 | 0.038 | 0.023 | 1.652 | 0.036 | 0.027 | 1.333 | 0.041 | 0.029 | 1.414 | 1.466 | 0.347 | 6 | 194 | 200 |
| 26 | TS00109407 2F | 0.065 | 0.053 | 1.226 | 0.071 | 0.060 | 1.183 | 0.070 | 0.052 | 1.346 | 1.252 | 0.703 | 3 | 197 | 200 |
| 27 | TS00148106 1 | 0.044 | 0.035 | 1.257 | 0.035 | 0.027 | 1.296 | 0.036 | 0.029 | 1.241 | 1.265 | 0.387 | 5 | 195 | 200 |
| 28 | TS00164406 3C | 0.031 | 0.024 | 1.292 | 0.025 | 0.023 | 1.087 | 0.029 | 0.021 | 1.381 | 1.253 | 0.303 | 7 | 193 | 200 |
| 29 | TS00187207 1 HED1D2 | 0.058 | 0.062 | 0.935 | 0.065 | 0.059 | 1.102 | 0.061 | 0.054 | 1.130 | 1.056 | 0.630 | 3 | 197 | 200 |
| 30 | TS00213305 1B | 0.031 | 0.030 | 1.033 | 0.029 | 0.033 | 0.879 | 0.035 | 0.031 | 1.129 | 1.014 | 0.343 | 6 | 194 | 200 |
| 31 | TS00232207 1 | 0.024 | 0.022 | 1.091 | 0.011 | 0.011 | 1.000 | 0.015 | 0.014 | 1.071 | 1.054 | 0.227 | 9 | 191 | 200 |
| 32 | TS00267305 3E | 0.095 | 0.082 | 1.159 | 0.086 | 0.087 | 0.989 | 0.096 | 0.089 | 1.079 | 1.075 | 0.857 | 2 | 198 | 200 |
| 33 | TS00315505 1G | 0.028 | 0.034 | 0.824 | 0.031 | 0.034 | 0.912 | 0.032 | 0.030 | 1.067 | 0.934 | 0.303 | 7 | 193 | 200 |
| 34 | TS00338107 2G | 0.056 | 0.026 | 2.154 | 0.057 | 0.018 | 3.167 | 0.024 | 0.020 | 1.200 | 2.174 | 0.563 | 4 | 196 | 200 |
| 35 | TS00486606 1A | 0.084 | 0.059 | 1.424 | 0.056 | 0.071 | 0.789 | 0.085 | 0.068 | 1.250 | 1.154 | 0.853 | 2 | 198 | 200 |
| 36 | TS00507307 2B | 0.046 | 0.033 | 1.394 | 0.048 | 0.018 | 2.667 | 0.043 | 0.031 | 1.387 | 1.816 | 0.457 | 4 | 196 | 200 |
| 37 | TS00624606 1 | 0.072 | 0.028 | 2.571 | 0.043 | 0.048 | 0.896 | 0.072 | 0.048 | 1.500 | 1.656 | 0.557 | 4 | 196 | 200 |
| 38 | TS00629005 1 | 0.081 | 0.076 | 1.066 | 0.095 | 0.072 | 1.319 | 0.091 | 0.083 | 1.096 | 1.161 | 0.833 | 2 | 198 | 200 |
| 39 | TS00727706 1 | 0.047 | 0.038 | 1.237 | 0.058 | 0.045 | 1.289 | 0.058 | 0.042 | 1.381 | 1.302 | 0.567 | 4 | 196 | 200 |
| 40 | TS00854605 3M | 0.048 | 0.027 | 1.778 | 0.047 | 0.032 | 1.469 | 0.037 | 0.029 | 1.276 | 1.507 | 0.483 | 4 | 196 | 200 |
| 41 | TS00858906 1C | 0.095 | 0.075 | 1.267 | 0.093 | 0.080 | 1.163 | 0.076 | 0.079 | 0.962 | 1.130 | 0.937 | 2 | 198 | 200 |
| 42 | TS00965006 3C | 0.057 | 0.028 | 2.036 | 0.058 | 0.039 | 1.487 | 0.053 | 0.039 | 1.359 | 1.627 | 0.577 | 3 | 197 | 200 |
| 43 | TS01129306 1B | 0.039 | 0.026 | 1.500 | 0.014 | 0.027 | 0.519 | 0.038 | 0.023 | 1.652 | 1.224 | 0.377 | 5 | 195 | 200 |
| 44 | TS01218406 1 | 0.064 | 0.065 | 0.985 | 0.057 | 0.057 | 1.000 | 0.055 | 0.062 | 0.887 | 0.957 | 0.697 | 3 | 197 | 200 |
| 45 | TS01292806 2K | 0.037 | 0.045 | 0.822 | 0.031 | 0.025 | 1.240 | 0.028 | 0.025 | 1.120 | 1.061 | 0.560 | 4 | 196 | 200 |
| 46 | TS01298705 1 | 0.020 | 0.041 | 0.488 | 0.028 | 0.039 | 0.718 | 0.033 | 0.040 | 0.825 | 0.677 | 0.520 | 4 | 196 | 200 |
| 47 | TS01353006 1 | 0.045 | 0.051 | 0.882 | 0.035 | 0.043 | 0.814 | 0.049 | 0.051 | 0.961 | 0.886 | 0.490 | 4 | 196 | 200 |
| 48 | TS01371006 1A | 0.037 | 0.034 | 1.088 | 0.031 | 0.026 | 1.192 | 0.036 | 0.034 | 1.059 | 1.113 | 0.293 | 7 | 193 | 200 |
| 49 | TS01388406 4F | 0.034 | 0.036 | 0.944 | 0.049 | 0.031 | 1.581 | 0.046 | 0.030 | 1.533 | 1.353 | 0.460 | 4 | 196 | 200 |
| 50 | TS01390006 1E | 0.074 | 0.068 | 1.088 | 0.077 | 0.074 | 1.041 | 0.077 | 0.073 | 1.055 | 1.061 | 0.773 | 3 | 197 | 200 |
| 51 | TS01399006 1 | 0.011 | 0.022 | 0.500 | 0.005 | 0.020 | 0.250 | 0.005 | 0.016 | 0.313 | 0.354 | 0.113 | 18 | 182 | 200 |
| 52 | TS01423506 3H | 0.040 | 0.028 | 1.429 | 0.034 | 0.025 | 1.360 | 0.036 | 0.026 | 1.385 | 1.391 | 0.260 | 8 | 192 | 200 |
| 53 | TS01514806 3D | 0.006 | 0.004 | 1.500 | 0.010 | 0.003 | 3.333 | 0.007 | 0.006 | 1.167 | 2.000 | 0.123 | 16 | 184 | 200 |
| 54 | TS01596705 1 | 0.008 | 0.016 | 0.500 | 0.011 | 0.018 | 0.611 | 0.007 | 0.019 | 0.368 | 0.493 | 0.147 | 14 | 186 | 200 |
| 55 | TS01898205 1 | 0.015 | 0.014 | 1.071 | 0.014 | 0.013 | 1.077 | 0.014 | 0.014 | 1.000 | 1.049 | 0.137 | 15 | 185 | 200 |
| 56 | TS01958205 1 | 0.010 | 0.009 | 1.111 | 0.007 | 0.010 | 0.700 | 0.007 | 0.008 | 0.875 | 0.895 | 0.067 | 30 | 170 | 200 |
| 57 | TS02131305 2D | 0.047 | 0.037 | 1.270 | 0.048 | 0.042 | 1.143 | 0.044 | 0.037 | 1.189 | 1.201 | 0.497 | 4 | 196 | 200 |
| 58 | TS02184105 2L | 0.036 | 0.027 | 1.333 | 0.041 | 0.028 | 1.464 | 0.041 | 0.019 | 2.158 | 1.652 | 0.350 | 6 | 194 | 200 |
| 59 | TS02338805 1 | 0.025 | 0.012 | 2.083 | 0.024 | 0.010 | 2.400 | 0.022 | 0.005 | 4.400 | 2.961 | 0.223 | 9 | 191 | 200 |
| 60 | TS02518405 1 | 0.002 | 0.007 | 0.286 | 0.007 | 0.011 | 0.636 | 0.011 | 0.004 | 2.750 | 1.224 | 0.103 | 19 | 181 | 200 |

# Appendix 14A

Positive PCR amplification results for β-globin and L1 of alpha HPV

| No | ID | Pathology code | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV status |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 01209199 2 | + | - | - | + | + |
| 2 | 2 | 954593 B | + | - | - | + | + |
| 3 | 3 | NS00196904 1 | + | - | + | + | + |
| 4 | 4 | NS00442204 1L | + | - | - | + | + |
| 5 | 6 | NS00671203 1 | + | - | + | + | + |
| 6 | 7 | NS00711302 1B | + | - | - | + | + |
| 7 | 8 | NS00848002 3D | + | - | - | + | + |
| 8 | 9 | NS01/68103 1A | + | - | - | + | + |
| 9 | 10 | NS01/68103 1B | + | - | - | + | + |
| 10 | 11 | NS01020203 3K | + | - | - | + | + |
| 11 | 12 | NS01029003 1 | + | - | - | + | + |
| 12 | 13 | NS01076404 1B | + | - | - | + | + |
| 13 | 15 | NS01291204 1C | + | - | - | + | + |
| 14 | 16 | NS01404702 1B | + | - | + | + | + |
| 15 | 22 | TS00012706 1 | + | - | - | + | + |
| 16 | 27 | TS00148106 1 | + | - | + | + | + |
| 17 | 32 | TS00267305 3E | + | - | - | + | + |
| 18 | 33 | TS00315505 1G | + | - | - | + | + |
| 19 | 37 | TS00624606 1 | + | - | + | + | + |
| 20 | 43 | TS01129306 1B | + | - | + | - | + |
| 21 | 45 | TS01292806 2K | + | + | + | + | + |
| 22 | 46 | TS01298705 1 | + | + | + | + | + |
| 23 | 47 | TS01353006 1 | + | + | + | + | + |
| 24 | 48 | TS01371006 1A | + | + | + | + | + |
| 25 | 49 | TS01388406 4F | + | + | + | + | + |
| 26 | 50 | TS01390006 1E | + | + | + | + | + |
| 27 | 52 | TS01423506 3H | + | + | + | + | + |
| 28 | 53 | TS01514806 3D | + | + | + | + | + |
| 29 | 54 | TS01596705 1 | + | + | + | + | + |
| 30 | 55 | TS01898205 1 | + | + | + | + | + |
| 31 | 56 | TS01958205 1 | + | + | + | + | + |
| 32 | 57 | TS02131305 2D | + | + | + | + | + |
| 33 | 58 | TS02184105 2L | + | + | + | + | + |
| 34 | 60 | TS02518405 1 | + | + | + | + | + |
| | | Total: | 34 | 14 | 20 | 33 (55.0) | 34 |
| | | Percentage: | 56.7 | 23.3 | 33.3 | 55.0 | 56.7 |
| | | Grand total: | 60 | 60 | 60 | 60 | 60 |

Note: + Positive PCR and - Negative PCR

## Appendix 14B

PCR amplification results for β-globin (positive) and L1 of α-HPV (negative)

| No | ID | Pathology code | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV status |
|---|---|---|---|---|---|---|---|
| 1 | 5 | NS00642004 1 | + | - | - | - | - |
| 2 | 14 | NS01258903 1C | + | - | - | - | - |
| 3 | 17 | NS01643002 1A | + | - | - | - | - |
| 4 | 18 | NS01803103 1 | + | - | - | - | - |
| 5 | 19 | NS01864704 1B | + | - | - | - | - |
| 6 | 20 | NS01960104 1 | + | - | - | - | - |
| 7 | 21 | NS02060804 1 | + | - | - | - | - |
| 8 | 23 | TS00044505 1 | + | - | - | - | - |
| 9 | 24 | TS00055407 1 | + | - | - | - | - |
| 10 | 25 | TS00059407 1 | + | - | - | - | - |
| 11 | 26 | TS00109407 2F | + | - | - | - | - |
| 12 | 28 | TS00164406 3C | + | - | - | - | - |
| 13 | 29 | TS00187207 1 HED1D2 | + | - | - | - | - |
| 14 | 30 | TS00213305 1B | + | - | - | - | - |
| 15 | 31 | TS00232207 1 | + | - | - | - | - |
| 16 | 34 | TS00338107 2G | + | - | - | - | - |
| 17 | 35 | TS00486606 1A | + | - | - | - | - |
| 18 | 36 | TS00507307 2B | + | - | - | - | - |
| 19 | 38 | TS00629005 1 | + | - | - | - | - |
| 20 | 39 | TS00727706 1 | + | - | - | - | - |
| 21 | 40 | TS00854605 3M | + | - | - | - | - |
| 22 | 41 | TS00858906 1C | + | - | - | - | - |
| 23 | 42 | TS00965006 3C | + | - | - | - | - |
| 24 | 44 | TS01218406 1 | + | - | - | - | - |
| 25 | 51 | TS01399006 1 | + | - | - | - | - |
| 26 | 59 | TS02338805 1 | + | - | - | - | - |
| | | Total: | 26 | - | - | - | 26 |
| | | Percentage: | 43.3 | | | | 43.3 |
| | | Grand total: | 60 | | | | 60 |

Note: + Positive PCR and - Negative PCR

# Appendix 15

## DNA quantification of the PCR products prior to automated DNA sequencing

| No. | Sample no. (tube no.) | PCR Methods[α] | Purification Methods[β] | Amplicon size (bp) | DNA purity $A_{260}/A_{280}$ | DNA yield (ηg/μl) | Volume (μl) | | 10 ηg/μl (μl) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | DNA | $dH_2O$ | |
| 1 | 45 (Tube #89) | iPCR | B | **450** | 1.061 | 55 | **4** | **16** | 20 |
| 2 | 46 (Tube #91) | iPCR | B | 450 | 0.677 | 140 | **1** | **19** | 20 |
| 3 | 47 (Tube #93) | iPCR | A | 450 | 0.886 | 60 | **3** | **17** | 20 |
| 4 | 48 (Tube #95) | iPCR | B | 450 | 1.113 | 35 | **6** | **14** | 20 |
| 5 | 49 (Tube #97) | iPCR | A | 450 | 1.353 | 95 | **2** | **18** | 20 |
| 6 | 50 (Tube #99) | iPCR | B | 450 | 1.061 | 40 | **5** | **15** | 20 |
| 7 | 52 (Tube #103) | iPCR | B | 450 | 1.391 | 40 | **5** | **15** | 20 |
| 8 | 53 (Tube #105) | iPCR | B | 450 | 2.000 | 40 | **5** | **15** | 20 |
| 9 | 54 (Tube #107) | iPCR | A | 450 | 0.493 | 30 | **7** | **13** | 20 |
| 10 | 55 (Tube #109) | iPCR | B | 450 | 1.049 | 30 | **7** | **13** | 20 |
| 11 | 56 (Tube #111) | iPCR | A | 450 | 0.895 | 50 | **4** | **16** | 20 |
| 12 | 57 (Tube #114) | iPCR | A | 450 | 1.201 | 45 | **4** | **16** | 20 |
| 13 | 58 (Tube #115) | iPCR | A | 450 | 1.652 | 85 | **2** | **18** | 20 |
| 14 | 60 (Tube #119) | iPCR | A | 450 | 1.224 | 55 | **4** | **16** | 20 |
| 15 | 3 (Tube #5) | iPCR | B | **190** | 1.032 | 25 | **8** | **12** | 20 |
| 16 | 6 (Tube #11) | iPCR | B | 190 | 1.079 | 30 | **7** | **13** | 20 |
| 17 | 16 (Tube #31) | iPCR | B | 190 | 1.492 | 15 | **13** | **7** | 20 |
| 18 | 27 (Tube #53) | iPCR | B | 190 | 1.265 | 62 | **3** | **17** | 20 |
| 19 | 37 (Tube #73) | iPCR | B | 190 | 1.656 | 85 | **2** | **18** | 20 |
| 20 | 43 (Tube #85) | iPCR | B | 190 | 1.224 | 60 | **3** | **17** | 20 |
| 21 | 45 (Tube #89) | snPCR | A | 190 | 1.421 | 125 | **2** | **18** | 20 |
| 22 | 46 (Tube #91) | snPCR | A | 190 | 1.070 | 165 | **1** | **19** | 20 |
| 23 | 47 (Tube #93) | snPCR | A | 190 | 0.886 | 120 | **2** | **18** | 20 |
| 24 | 48 (Tube #95) | snPCR | A | 190 | 0.910 | 145 | **1** | **19** | 20 |
| 25 | 49 (Tube #97) | snPCR | A | 190 | 0.925 | 140 | **1** | **19** | 20 |
| 26 | 50 (Tube #99) | snPCR | A | 190 | 1.140 | 135 | **1** | **19** | 20 |
| 27 | 52 (Tube #103) | snPCR | A | 190 | 1.541 | 150 | **1** | **19** | 20 |
| 28 | 53 (Tube #105) | snPCR | A | 190 | 2.361 | 150 | **1** | **19** | 20 |
| 29 | 54 (Tube #107) | snPCR | A | 190 | 1.132 | 185 | **1** | **19** | 20 |
| 30 | 55 (Tube #109) | snPCR | A | 190 | 0.936 | 80 | **2** | **18** | 20 |
| 31 | 56 (Tube #111) | snPCR | A | 190 | 1.044 | 70 | **3** | **17** | 20 |
| 32 | 57 (Tube #114) | snPCR | A | 190 | 1.270 | 70 | **3** | **17** | 20 |
| 33 | 58 (Tube #115) | snPCR | A | 190 | 1.500 | 70 | **3** | **17** | 20 |
| 34 | 60 (Tube #119) | snPCR | A | 190 | 1.032 | 95 | **2** | **18** | 20 |
| 35 | 1 (Tube #1) | iPCR | B | **140** | 0.980 | 80 | **3** | **17** | 20 |
| 36 | 2 (Tube #3) | iPCR | B | 140 | 1.257 | 55 | **4** | **16** | 20 |
| 37 | 3 (Tube #5) | iPCR | B | 140 | 1.115 | 55 | **4** | **16** | 20 |
| 38 | 4 (Tube #7) | iPCR | B | 140 | 1.475 | 45 | **4** | **16** | 20 |
| 39 | 6 (Tube #11) | iPCR | B | 140 | 1.485 | 30 | **7** | **13** | 20 |
| 40 | 7 (Tube #13) | iPCR | B | 140 | 2.125 | 15 | **13** | **7** | 20 |
| 41 | 8 (Tube #15) | iPCR | B | 140 | 1.412 | 95 | **2** | **18** | 20 |
| 42 | 9 (Tube #17) | iPCR | B | 140 | 1.125 | 20 | **10** | **10** | 20 |
| 43 | 10 (Tube #20) | iPCR | B | 140 | 1.333 | 20 | **10** | **10** | 20 |
| 44 | 11 (Tube #21) | iPCR | B | 140 | 1.109 | 35 | **6** | **14** | 20 |
| 45 | 12 (Tube #23) | iPCR | B | 140 | 1.098 | 55 | **4** | **16** | 20 |
| 46 | 13 (Tube #25) | iPCR | B | 140 | 1.275 | 75 | **3** | **17** | 20 |
| 47 | 15 (Tube #30) | iPCR | B | 140 | 1.262 | 30 | **7** | **13** | 20 |
| 48 | 16 (Tube #31) | iPCR | B | 140 | 2.143 | 60 | **3** | **17** | 20 |
| 49 | 22 (Tube #43) | iPCR | B | 140 | 1.039 | 25 | **8** | **12** | 20 |
| 50 | 27 (Tube #53) | iPCR | B | 140 | 1.297 | 55 | **4** | **16** | 20 |
| 51 | 32 (Tube #63) | iPCR | B | 140 | 1.269 | 40 | **5** | **15** | 20 |
| 52 | 33 (Tube #65) | iPCR | B | 140 | 1.100 | 130 | **2** | **18** | 20 |
| 53 | 37 (Tube #73) | iPCR | B | 140 | 1.500 | 205 | **1** | **19** | 20 |
| 54 | 45 (Tube #80) | nPCR | A | 140 | 1.547 | 150 | **1** | **19** | 20 |
| 55 | 46 (Tube #91) | nPCR | A | 140 | 1.089 | 145 | **1** | **19** | 20 |
| 56 | 47 (Tube #93) | nPCR | A | 140 | 0.961 | 145 | **1** | **19** | 20 |
| 57 | 48 (Tube #95) | nPCR | A | 140 | 1.192 | 105 | **2** | **18** | 20 |
| 58 | 49 (Tube #97) | nPCR | A | 140 | 1.581 | 105 | **2** | **18** | 20 |
| 59 | 50 (Tube #99) | nPCR | A | 140 | 1.318 | 120 | **2** | **18** | 20 |
| 60 | 52 (Tube #103) | nPCR | A | 140 | 1.429 | 100 | **2** | **18** | 20 |
| 61 | 53 (Tube #105) | nPCR | A | 140 | 1.333 | 85 | **2** | **18** | 20 |
| 62 | 54 (Tube #107) | nPCR | A | 140 | 1.167 | 80 | **3** | **17** | 20 |
| 63 | 55 (Tube #109) | nPCR | A | 140 | 1.077 | 80 | **3** | **17** | 20 |
| 64 | 56 (Tube #111) | nPCR | A | 140 | 1.188 | 80 | **3** | **17** | 20 |
| 65 | 57 (Tube #114) | nPCR | A | 140 | 1.189 | 90 | **2** | **18** | 20 |
| 66 | 58 (Tube #115) | nPCR | A | 140 | 1.464 | 110 | **2** | **18** | 20 |
| 67 | 60 (Tube #119) | nPCR | A | 140 | 1.125 | 100 | **2** | **18** | 20 |

Notes:

*α* - PCR methods: iPCR – Independent PCR; snPCR - Semi-nested PCR; and nPCR - Nested PCR

*β* - PCR product purification methods: A - QIAquick PCR Purification Kit (Qiagen); B - QIAquick Gel Extraction Kit (Qiagen)

# Appendix 16

## Automated DNA sequencing results (actual size obtained from contig)

| No. | Sample no. (tube no.) | PCR Methods[a] | Purification Methods[b] | Expected amplicon size (bp) | Actual amplicon size size (bp) |
|---|---|---|---|---|---|
| 1 | 45 (Tube #89) | iPCR | B | **450** | 422 |
| 2 | 46 (Tube #91) | iPCR | B | 450 | 447 |
| 3 | 47 (Tube #93) | iPCR | A | 450 | 453 |
| 4 | 48 (Tube #95) | iPCR | B | 450 | 451 |
| 5 | 49 (Tube #97) | iPCR | A | 450 | 452 |
| 6 | 50 (Tube #99) | iPCR | B | 450 | 454 |
| 7 | 52 (Tube #103) | iPCR | B | 450 | 444 |
| 8 | 53 (Tube #105) | iPCR | B | 450 | 447 |
| 9 | 54 (Tube #107) | iPCR | A | 450 | 450 |
| 10 | 55 (Tube #109) | iPCR | B | 450 | 451 |
| 11 | 56 (Tube #111) | iPCR | A | 450 | 451 |
| 12 | 57 (Tube #114) | iPCR | A | 450 | 451 |
| 13 | 58 (Tube #115) | iPCR | A | 450 | 451 |
| 14 | 60 (Tube #119) | iPCR | A | 450 | 447 |
| 15 | 3 (Tube #5) | iPCR | B | **190** | 183 |
| 16 | 6 (Tube #11) | iPCR | B | 190 | 179 |
| 17 | 16 (Tube #31) | iPCR | B | 190 | 205 |
| 18 | 27 (Tube #53) | iPCR | B | 190 | 181 |
| 19 | 37 (Tube #73) | iPCR | B | 190 | 179 |
| 20 | 43 (Tube #85) | iPCR | B | 190 | 180 |
| 21 | 45 (Tube #89) | snPCR | A | 190 | 146 (partial) |
| 22 | 46 (Tube #91) | snPCR | A | 190 | 147 (partial) |
| 23 | 47 (Tube #93) | snPCR | A | 190 | 181 |
| 24 | 48 (Tube #95) | snPCR | A | 190 | 190 |
| 25 | 49 (Tube #97) | snPCR | A | 190 | 145 (partial) |
| 26 | 50 (Tube #99) | snPCR | A | 190 | 144 (partial) |
| 27 | 52 (Tube #103) | snPCR | A | 190 | 143 (partial) |
| 28 | 53 (Tube #105) | snPCR | A | 190 | 152 (partial) |
| 29 | 54 (Tube #107) | snPCR | A | 190 | 144 (partial) |
| 30 | 55 (Tube #109) | snPCR | A | 190 | 181 |
| 31 | 56 (Tube #111) | snPCR | A | 190 | 153 (partial) |
| 32 | 57 (Tube #114) | snPCR | A | 190 | 141 (partial) |
| 33 | 58 (Tube #115) | snPCR | A | 190 | 144 (partial) |
| 34 | 60 (Tube #119) | snPCR | A | 190 | 148 (partial) |
| 35 | 1 (Tube #1) | iPCR | B | **140** | 141 |
| 36 | 2 (Tube #3) | iPCR | B | 140 | 141 |
| 37 | 3 (Tube #5) | iPCR | B | 140 | 141 |
| 38 | 4 (Tube #7) | iPCR | B | 140 | 141 |
| 39 | 6 (Tube #11) | iPCR | B | 140 | 141 |
| 40 | 7 (Tube #13) | iPCR | B | 140 | 141 |
| 41 | 8 (Tube #15) | iPCR | B | 140 | 141 |
| 42 | 9 (Tube #17) | iPCR | B | 140 | 141 |
| 43 | 10 (Tube #20) | iPCR | B | 140 | 141 |
| 44 | 11 (Tube #21) | iPCR | B | 140 | 141 |
| 45 | 12 (Tube #23) | iPCR | B | 140 | 141 |
| 46 | 13 (Tube #25) | iPCR | B | 140 | 141 |
| 47 | 15 (Tube #30) | iPCR | B | 140 | 141 |
| 48 | 16 (Tube #31) | iPCR | B | 140 | 141 |
| 49 | 22 (Tube #43) | iPCR | B | 140 | 141 |
| 50 | 27 (Tube #53) | iPCR | B | 140 | 141 |
| 51 | 32 (Tube #63) | iPCR | B | 140 | 141 |
| 52 | 33 (Tube #65) | iPCR | B | 140 | 141 |
| 53 | 37 (Tube #73) | iPCR | B | 140 | 145 |
| 54 | 45 (Tube #80) | nPCR | A | 140 | 141 |
| 55 | 46 (Tube #91) | nPCR | A | 140 | 141 |
| 56 | 47 (Tube #93) | nPCR | A | 140 | 141 |
| 57 | 48 (Tube #95) | nPCR | A | 140 | 147 |
| 58 | 49 (Tube #97) | nPCR | A | 140 | 141 |
| 59 | 50 (Tube #99) | nPCR | A | 140 | 141 |
| 60 | 52 (Tube #103) | nPCR | A | 140 | 139 |
| 61 | 53 (Tube #105) | nPCR | A | 140 | 139 |
| 62 | 54 (Tube #107) | nPCR | A | 140 | 139 |
| 63 | 55 (Tube #109) | nPCR | A | 140 | 139 |
| 64 | 56 (Tube #111) | nPCR | A | 140 | 139 |
| 65 | 57 (Tube #114) | nPCR | A | 140 | 138 |
| 66 | 58 (Tube #115) | nPCR | A | 140 | 140 |
| 67 | 60 (Tube #119) | nPCR | A | 140 | 138 |

Notes:

*a* - PCR methods: iPCR – Independent PCR; snPCR - Semi-nested PCR; and nPCR - Nested PCR

*b* - PCR product purification methods: A - QIAquick PCR Purification Kit (Qiagen); B - QIAquick Gel Extraction Kit (Qiagen)

# Appendix 17

## CLUSTAL W (1.83) multiple sequences alignment of L1 HPVs

```
45_422 (sample) --------------CCAAAAGGATACTGATCCAATTCACTAGAAAACTTTTCTTTTAAAT
hsg_391         ----------TCGTCCAAAAGGATACTGATCTAAGTCTAAAGAAAACTTTTCCTTTAAAT
HPV16           AGTGGTTCTATGGTTACCTCTGATGCCCAAATATTCAATAAACCTTATTGGTTACAACGA
HPV6            AGCGGCTCTTTGGTGTCCTCTGAGGCACAATTGTTTAATAAGCCATATTGGCTACAAAAA
HPV18           AGTGGCTCTATTGTTACCTCTGACTCCCAGTTGTTTAATAAACCATATTGGTTACATAAG

45_422 (sample) TAACCTCCCAAAAACTAAGGTTCTTATAGGGATC-TGGCTTTTCCTTTTCAGGAGTGGGC
hsg_391         CCACATTCCAAAACTTTAACTTATCATAGGGATC-CTTATTTTCAGCCGGTGCAGCATCC
HPV16           GCACAGGGCCACAATAATGGCATTTGTTGGGGTAACCAACTATTTGTTACTGTTGTTGAT
HPV6            GCCCAGGGACATAACAATGGTATTTGTTGGGGTAATCAACTGTTTGTTACTGTGGTAGAT
HPV18           GCACAGGGTCATAACAATGGTGTTTGCTGGCATAATCAATTATTTGTTACTGTGGTAGAT

45_422 (sample) TTTTGACA-GGTAATGGCCTGTGACTGCACATACCTATAGGTATCTTCTAATGTACC--A
hsg_391         TTTTGACA-GGTAATAGCAACAGATTGTACAAAACGATATGTATCCACCAAACTAGT--A
HPV16           ACTACACGCAGTACAAATATGTCATTATGTGCTGCCATATCTACTTCAGAAACTAC---A
HPV6            ACCACACGCAGTACCAACATGACATTATGTGCATCCGTAACTACATCTT---CCAC---A
HPV18           ACCACTCCCAGTACCAATTTAACAATATGTGCTTCTACACAGTCTCCTGTACCTGGGCAA

45_422 (sample) TTTGGGGGAGGCGATAACCCAAAGTTCC--AGTCTTCCAAAACAGAGGGATTC-------
hsg_391         GTTGGCGGGGGGGGAACACCAAAGTTCC--AATCCTCTAAAATACTGCTATTC-------
HPV16           TATAAAAATACTAACTTTAAGGAGTACCTACGACATGGGGAGGAATATGATTTACAGTTT
HPV6            TACACCAATTCTGATTATAAAGAGTACATGCGTCATGTGGAAGAGTATGATTTACAATTT
HPV18           TATGATGCTACCAAATTTAAGCAGTATAGCAGACATGTTGAGGAATATGATTTGCAGTTT
                                                             *

45_422 (sample) ATTGTGTGAATATAGGCCATTACTTCAGCAGACAATGTAATGCTACATAATTGAAAAATA
hsg_391         ATACTATGAATATAGGACATAACATCTGCAGTTAAAGTAATAGTACACAACTGAAAAATA
HPV16           ATTTTTCAACTGTGCAAAATAACCTTAACTGCAGACGTTATGACATACATACATTCTATG
HPV6            ATTTTTCAATTATGTAGCATTACATTGTCTGCTGAAGTAATGGCCTATATTCACACAATG
HPV18           ATTTTTCAGTTGTGTACTATTACTTTAACTGCAGATGTTATGTCCTATATTCATAGTATG
                    *     * *      * **     * *   * ** **     * *        **

45_422 (sample) AATTGTA-AATCATACTCTTCCACATGACGCATGTACTCTTTATAATCAGAATTGGTGTA
hsg_391         AACTGCA-AATCATATTCCTCAACATGTCTGCTATACTGCTTAAATTTGGTAGCATCATA
HPV16           AATTCCACTATTTTGGAGGACTGGAATTTTGGTCTACAACCTCCCCCAGGAGGCACACTA
HPV6            AATCCCTCTGTTTTGGAAGACTGGAACTTTGGGTTATCGCCTCCCCCAAATGGTACATTA
HPV18           AATAGCAGTATTTTAGAGGATTGGAACTTTGGTGTTCCCCCCCCCCCAACTACTAGTTTG
                          *

45_422 (sample) TGTGGAAGATGTAGT------TACGGATGCACATAATGTCATGTTGGTACTGCGTGTGGT
hsg_391         TTGCCCAGGTACAGGAGACTGTGTAGAAGCACATATTGTTAAATTGGTACTGCGA-----
HPV16           GAAGATACTTATAGG----TTTGTAACC---CAGGCAATTGCTTGTCAAAAACATACACC
HPV6            GAAGATACCTATAGG----TATGTGCAGTCACAGGCCATTACCTGTCAAAAGCCCA---C
HPV18           GTGGATACATATCGT----TTTGTACAATCTGTTGCTATTACCTGTCAAAAGGATG---C

45_422 (sample) ATCTACCACAGTAACAAACAGTTGATTACCCCAACA------------------------
hsg_391         ------------------------------------------------------------
HPV16           TCCAGCACCTAAAGAAGATGATCCCCTTAAAAAATACACTTTTTGGGAAGTAAATTTAAA
HPV6            TCCTGAAAAGGAAAAGCCAGATCCCTATAAGAACCTTAGTTTTTGGGAGGTTAATTTAAA
HPV18           TGCACCGGCTGAAAATAAGGATCCCTATGATAAGTTAAAGTTTTGGAATGTGGATTTAAA

45_422 (sample) ------------------------------------------------------------
hsg_391         ------------------------------------------------------------
HPV16           GGAAAAGTTTTCTGCAGACCTAGATCAGTTTCCTTTAGGACGCAAATTTTTACTACAAGC
HPV6            AGAAAAGTTTTCTAGTGAATTGGATCAGTATCCTTTGGGACGCAAGTTTTTGTTACAAAG
HPV18           GGAAAAGTTTTCTTTAGACTTAGATCAATATCCCCTTGGACGTAAATTTTTGGTTCAGGC
```

Note:

* - To denote nucleotide/s which are identical in all DNA sequences compared (sample and controls)

# Appendix 18

Results of the DNA sequencing alignment of L1 α-HPV

| No | ID | Pathology code | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|
| 1 | 1 | 01209199 2 | - | - | HPV6 | HPV6 |
| 2 | 2 | 954593 B | - | - | HPV6 | HPV6 |
| 3 | 3 | NS00196904 1 | - | HPV6 | HPV6 | HPV6 |
| 4 | 4 | NS00442204 1L | - | - | HPV6 | HPV6 |
| 5 | 6 | NS00671203 1 | - | HPV6 | HPV6 | HPV6 |
| 6 | 7 | NS00711302 1B | - | - | HPV6 | HPV6 |
| 7 | 8 | NS00848002 3D | - | - | HPV6 | HPV6 |
| 8 | 9 | NS01/68103 1A | - | - | HPV6 | HPV6 |
| 9 | 10 | NS01/68103 1B | - | - | HPV6 | HPV6 |
| 10 | 11 | NS01020203 3K | - | - | HPV6 | HPV6 |
| 11 | 12 | NS01029003 1 | - | - | HPV6 | HPV6 |
| 12 | 13 | NS01076404 1B | - | - | HPV6 | HPV6 |
| 13 | 15 | NS01291204 1C | - | - | HPV6 | HPV6 |
| 14 | 16 | NS01404702 1B | - | HPV6 | HPV6 | HPV6 |
| 15 | 22 | TS00012706 1 | - | - | HPV6 | HPV6 |
| 16 | 27 | TS00148106 1 | - | HPV16 | HPV16 | HPV16 |
| 17 | 32 | TS00267305 3E | - | - | HPV6 | HPV6 |
| 18 | 33 | TS00315505 1G | - | - | HPV6 | HPV6 |
| 19 | 37 | TS00624606 1 | - | HPV35 | HPV35 | HPV35 |
| 20 | 43 | TS01129306 1B | - | HPV6 | - | HPV6 |
| 21 | 45 | TS01292806 2K | HPV6 | HPV6 | HPV6 | HPV6 |
| 22 | 46 | TS01298705 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| 23 | 47 | TS01353006 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| 24 | 48 | TS01371006 1A | HPV6 | HPV6 | HPV6 | HPV6 |
| 25 | 49 | TS01388406 4F | HPV6 | HPV6 | HPV6 | HPV6 |
| 26 | 50 | TS01390006 1E | HPV6 | HPV6 | HPV6 | HPV6 |
| 27 | 52 | TS01423506 3H | HPV6 | HPV6 | HPV6 | HPV6 |
| 28 | 53 | TS01514806 3D | HPV6 | HPV6 | HPV6 | HPV6 |
| 29 | 54 | TS01596705 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| 30 | 55 | TS01898205 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| 31 | 56 | TS01958205 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| 32 | 57 | TS02131305 2D | HPV6 | HPV6 | HPV6 | HPV6 |
| 33 | 58 | TS02184105 2L | HPV6 | HPV6 | HPV6 | HPV6 |
| 34 | 60 | TS02518405 1 | HPV6 | HPV6 | HPV6 | HPV6 |
| | | Total: | 14 | 20 | 33 | 34 |
| | | Percentage: | 23.3 | 33.3 | 55.0 | 56.7 |
| | | Grand total: | 60 | 60 | 60 | 60 |

Note: - Negative PCR

# Appendix 19

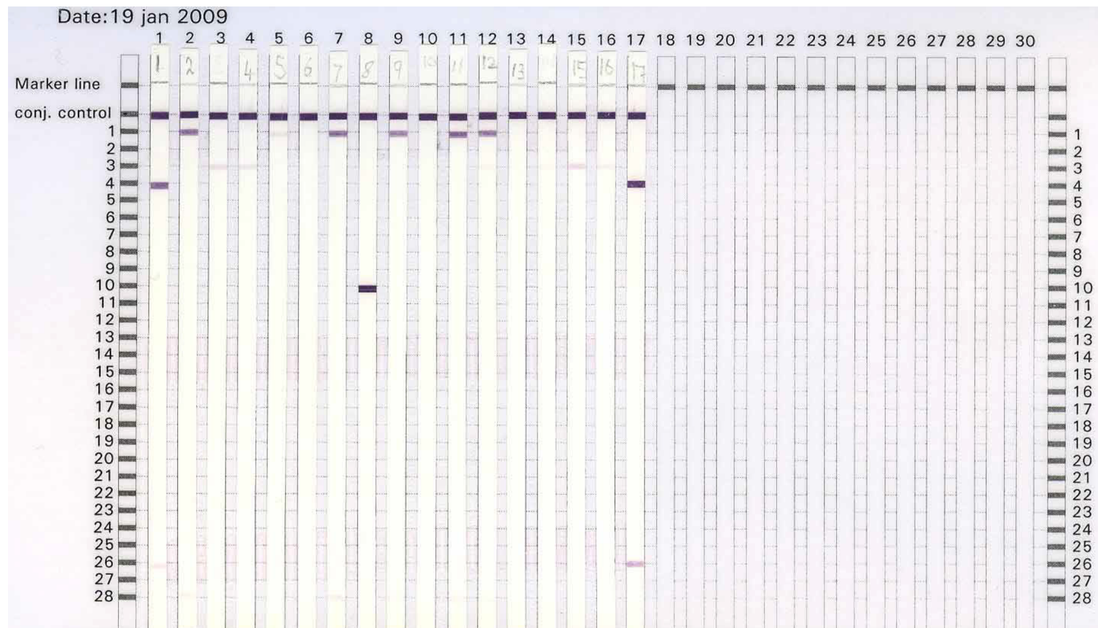
α-HPV genotyping: Raw data of SPF-DEIA analysis (microtiter plate layout)

PROBE1A;09DERD002;09/01/19;14/24;
1
450

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| A | 3.260 | 0.085 | 0.087 | 2.716 | 0.088 | 0.094 | 1.899 | 0.131 |
| B | 0.531 | 0.079 | 0.076 | 0.078 | 0.083 | 0.084 | 0.085 | 0.089 |
| C | 0.088 | 0.078 | 0.827 | 0.072 | 0.072 | 0.073 | 0.078 | 1.135 |
| D | 1.751 | 0.078 | 0.074 | 0.074 | 0.075 | 0.080 | 1.299 | 3.125 |
| E | 2.070 | 1.956 | 0.069 | 0.072 | 0.072 | 0.074 | 2.945 | 1.715 |
| F | 0.087 | 0.198 | 0.071 | 0.070 | 0.070 | 0.087 | 2.954 | 0.082 |
| G | 2.301 | 0.074 | 0.071 | 0.073 | 0.072 | 0.073 | 0.074 | 0.079 |
| H | 0.101 | 0.079 | 0.076 | 2.182 | 2.580 | 0.084 | 0.079 | 2.677 |

## Appendix 20

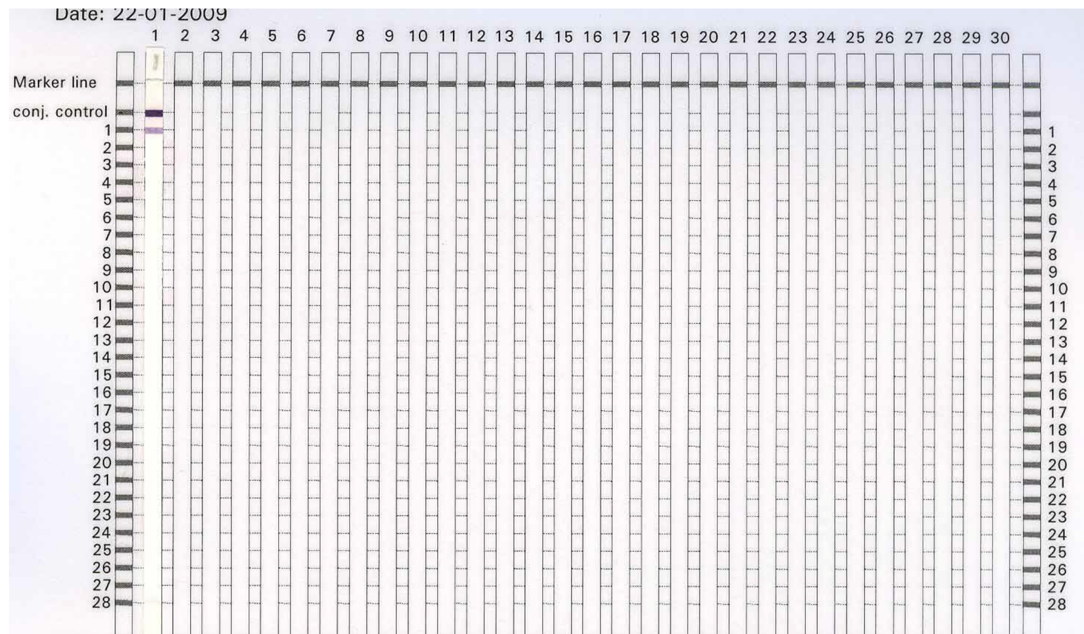
### α-HPV genotyping: Raw data of $SPF_{10}$-$LiPA_{25}$ (version 1)




| LiPA strip | Sample ID | Reactive probes | Genotyping results | Remark |
|---|---|---|---|---|
| 1 | 1 (Tube #1) | 4,26 | HPV18 | |
| 2 | 2 (Tube #3) | 1,28 | HPV6 | |
| 3 | 4 (Tube #7) | 3 | HPV16 | |
| 4 | 10 (Tube #20) | 3 | HPV16 | |
| 5 | 11 (Tube #21) | 1 | HPV6 | DEIA negative but elevated |
| 6 | 22 (Tube #43) | - | X | |
| 7 | 29 (Tube #57) | 1,28 | HPV6 | |
| 8 | 37 (Tube #73) | 10 | HPV35 | |
| 9 | 46 (Tube #91) | 1,28 | HPV6 | |
| 10 | 49 (Tube #97) | - | X | |
| 11 | 50 (Tube #99) | 1,28 | HPV6 | |
| 12 | 51 (Tube #101) | 1,3,28 | HPV6,HPV16 | |
| 13 | 54 (Tube #107) | - | Negative | DEIA negative but elevated |
| 14 | 56 (Tube #111) | 3 | HPV16 | |
| 15 | 57 (Tube #114) | 3 | HPV16 | |
| 16 | 58 (Tube #115) | 3 | HPV16 | |
| 17 | PCR control (+) | 4,26 | HPV18 | |

## Appendix 20 (Continued)

α-HPV genotyping: Raw data of $SPF_{10}$- $LiPA_{25}$ (version 1)




| LiPA strip | Sample ID | Reactive probes | Genotyping results | Remark |
|---|---|---|---|---|
| 1 | 16 (Tube #31) | 1,28 | HPV6 | |

# Appendix 21

Interpretation sheet for α-HPV genotyping: $SPF_{10}$- $LiPA_{25}$ (version 1)

| | | HPV genotypes | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| probe line | probe | 6 | 6 | 11 | 16 | 18 | 18 | 18 | 18 | 31 | 33 | 34 | 35 | 39 | 39 or 68 or 73 | 40 | 42 | 43 | 44 | 44 | 45 | 45 | 51 | 52 | 53 | 54 | 56 | 58 | 58 | 59 | 66 | 68 or 73 | 70 | 74 | 74 |
| 1 | 6 | X | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 11 | | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 16 | | | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 18 | | | | | X | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 18b | | | | | | | X | X | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 31/40/58 | | | | | | | | | X | | | | | | X | | | | | | | | | | | | X | X | | | | | | |
| 7 | c*31/33/54 | | | | | | | | | X | X | | | | | | | | | | | | | | | X | | | | | | | | | |
| 8 | c*33 | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 | 34 | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | 35 | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | | | |
| 11 | 39 | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | | | |
| 12 | 40 | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | | |
| 13 | 42 | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | | |
| 14 | 43 | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | | | | | | |
| 15 | 44 | | | | | | | | | | | | | | | | | | X | X | | | | | | | | | | | | | | | |
| 16 | 45 | | | | | | | | | | | | | | | | | | | | X | X | | | | | | | | | | | | | |
| 17 | 51 | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | | |
| 18 | 52 | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | | |
| 19 | 53 | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | |
| 20 | 56/74 | | | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | | | X | X |
| 21 | c*56/44/58 | | | | | | | | | | | | | | | | | | | X | | | | | | | X | | X | | | | | | |
| 22 | c*58 | | | | | | | | | | | | | | | | | | | | | | | | | | | X | X | | | | | | |
| 23 | 59 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | X | | | | | |
| 24 | 66 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | X | | | | |
| 25 | 68/45 | | | | | | | | | | | | | | | | | | | | | X | | | | | | | | | | X | | | |
| 26 | c*68/18/39 | | | | | | X | | X | | | | | X | X | | | | | | | | | | | | | | | | | X | | | |
| 27 | 70 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | X | | |
| 28 | 74/6 | | X | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | X |

Remark:
type 68 should be read as type 68 or 73
c*=confirmation

Note:-

Reproduced with written permission from DDL, Voorburg, The Netherlands

# Appendix 22

## Alpha HPV genotyping by using $SPF_{10}$-$LiPA_{25}$ (version 1)

| Sample no. | Pathology code | SPF-DEIA Plate | SPF-DEIA OD | SPF-DEIA Status | $SPF_{10}$-LiPA | $SPF_{10}$-$LiPA_{25}$ status |
|---|---|---|---|---|---|---|
| 1 | 01209199 2 | D1 | 1.751 | + | HPV18 | HPV18 |
| 2 | 954593 B | E1 | 2.070 | + | HPV6 | HPV6 |
| 3 | NS00196904 1 | F1 | 0.087 | - | - | - |
| 4 | NS00442204 1L | G1 | 2.301 | + | HPV16 | HPV16 |
| 5 | NS00642004 1 | H1 | 0.101 | - | - | - |
| 6 | NS00671203 1 | A2 | 0.085 | - | - | - |
| 7 | NS00711302 1B | B2 | 0.079 | - | - | - |
| 8 | NS00848002 3D | C2 | 0.078 | - | - | - |
| 9 | NS01/68103 1A | D2 | 0.078 | - | - | - |
| 10 | NS01/68103 1B | E2 | 1.956 | + | HPV16 | HPV16 |
| 11 | NS01020203 3K | F2 | 0.198 | + | HPV6 | HPV6 |
| 12 | NS01029003 1 | G2 | 0.074 | - | - | - |
| 13 | NS01076404 1B | H2 | 0.079 | - | - | - |
| 14 | NS01258903 1C | A3 | 0.087 | - | - | - |
| 15 | NS01291204 1C | B3 | 0.076 | - | - | - |
| 16 | NS01404702 1B | C3 | 0.827 | + | HPV6 | HPV6 |
| 17 | NS01643002 1A | D3 | 0.074 | - | - | - |
| 18 | NS01803103 1 | E3 | 0.069 | - | - | - |
| 19 | NS01864704 1B | F3 | 0.071 | - | - | - |
| 20 | NS01960104 1 | G3 | 0.071 | - | - | - |
| 21 | NS02060804 1 | H3 | 0.076 | - | - | - |
| 22 | TS00012706 1 | A4 | 2.716 | + | N/I | N/I |
| 23 | TS00044505 1 | B4 | 0.078 | - | - | - |
| 24 | TS00055407 1 | C4 | 0.072 | - | - | - |
| 25 | TS00059407 1 | D4 | 0.074 | - | - | - |
| 26 | TS00109407 2F | E4 | 0.072 | - | - | - |
| 27 | TS00148106 1 | F4 | 0.070 | - | - | - |
| 28 | TS00164406 3C | G4 | 0.073 | - | - | - |
| 29 | TS00187207 1 | H4 | 2.182 | + | HPV6 | HPV6 |
| 30 | TS00213305 1B | A5 | 0.088 | - | - | - |
| 31 | TS00232207 1 | B5 | 0.083 | - | - | - |
| 32 | TS00267305 3E | C5 | 0.072 | - | - | - |
| 33 | TS00315505 1G | D5 | 0.075 | - | - | - |
| 34 | TS00338107 2G | E5 | 0.072 | - | - | - |
| 35 | TS00486606 1A | F5 | 0.070 | - | - | - |
| 36 | TS00507307 2B | G5 | 0.072 | - | - | - |
| 37 | TS00624606 1 | H5 | 2.580 | + | HPV35 | HPV35 |
| 38 | TS00629005 1 | A6 | 0.094 | - | - | - |
| 39 | TS00727706 1 | B6 | 0.084 | - | - | - |
| 40 | TS00854605 3M | C6 | 0.073 | - | - | - |
| 41 | TS00858906 1C | D6 | 0.080 | - | - | - |
| 42 | TS00965006 3C | E6 | 0.074 | - | - | - |
| 43 | TS01129306 1B | F6 | 0.087 | - | - | - |
| 44 | TS01218406 1 | G6 | 0.073 | - | - | - |
| 45 | TS01292806 2K | H6 | 0.084 | - | - | - |
| 46 | TS01298705 1 | A7 | 1.899 | + | HPV6 | HPV6 |
| 47 | TS01353006 1 | B7 | 0.085 | - | - | - |
| 48 | TS01371006 1A | C7 | 0.078 | - | - | - |
| 49 | TS01388406 4F | D7 | 1.299 | + | N/I | N/I |
| 50 | TS01390006 1E | E7 | 2.945 | + | HPV6 | HPV6 |
| 51 | TS01399006 1 | F7 | 2.954 | + | HPV6/16 | HPV6/16 |
| 52 | TS01423506 3H | G7 | 0.074 | - | - | - |
| 53 | TS01514806 3D | H7 | 0.079 | - | - | - |
| 54 | TS01596705 1 | A8 | 0.131 | - | neg. | neg. but elevated |
| 55 | TS01898205 1 | B8 | 0.089 | - | - | - |
| 56 | TS01958205 1 | C8 | 1.135 | + | HPV16 | HPV16 |
| 57 | TS02131305 2D | D8 | 3.125 | + | HPV16 | HPV16 |
| 58 | TS02184105 2L | E8 | 1.715 | + | HPV16 | HPV16 |
| 59 | TS02338805 1 | F8 | 0.082 | - | - | - |
| 60 | TS02518405 1 | G8 | 0.079 | - | - | - |
| | Positive | A1 | 3.260 | | | |
| | Borderline | B1 | 0.531 | | | |
| | Negative | C1 | 0.088 | | | |
| | HPV18 | H8 | 2.677 | | | |
| | | | Positive | 16 | 16 | 16 |
| | | | Percentage | 26.7 | 26.7 | 26.7 |
| | | | Total | 60 | 60 | 60 |

Note: + positive, HPV present; - negative, HPV absent; N/I not identified

# Appendix 23

## Overall alpha HPV types detected by several methods in pilot study samples

| Sample no. | Pathology code | Low risk α-HPV | High risk α-HPV | | | α-HPV status |
|---|---|---|---|---|---|---|
| | | HPV6 | HPV16 | HPV18 | HPV35 | |
| 1 | 01209199 2 | $+^{\delta}$ | | $+^{\varepsilon}$ | | DI; HPV6, HPV18 |
| 2 | 954593 B | $+^{\delta,\varepsilon}$ | | | | SI; HPV6 |
| 3 | NS00196904 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 4 | NS00442204 1L | $+^{\delta}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 5 | NS00642004 1 | - | - | - | - | Negative |
| 6 | NS00671203 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 7 | NS00711302 1B | $+^{\delta}$ | | | | SI; HPV6 |
| 8 | NS00848002 3D | $+^{\delta}$ | | | | SI; HPV6 |
| 9 | NS01/68103 1A | $+^{\delta}$ | | | | SI; HPV6 |
| 10 | NS01/68103 1B | $+^{\delta}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 11 | NS01020203 3K | $+^{\delta,\varepsilon}$ | | | | SI; HPV6 |
| 12 | NS01029003 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 13 | NS01076404 1B | $+^{\delta}$ | | | | SI; HPV6 |
| 14 | NS01258903 1C | - | - | - | - | Negative |
| 15 | NS01291204 1C | $+^{\delta}$ | | | | SI; HPV6 |
| 16 | NS01404702 1B | $+^{\delta,\varepsilon}$ | | | | SI; HPV6 |
| 17 | NS01643002 1A | - | - | - | - | Negative |
| 18 | NS01803103 1 | - | - | - | - | Negative |
| 19 | NS01864704 1B | - | - | - | - | Negative |
| 20 | NS01960104 1 | - | - | - | - | Negative |
| 21 | NS02060804 1 | - | - | - | - | Negative |
| 22 | TS00012706 1 | $+^{\delta,\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | DI; HPV6; α-HPV untypable |
| 23 | TS00044505 1 | - | - | - | - | Negative |
| 24 | TS00055407 1 | - | - | - | - | Negative |
| 25 | TS00059407 1 | - | - | - | - | Negative |
| 26 | TS00109407 2F | - | - | - | - | Negative |
| 27 | TS00148106 1 | | $+^{\delta}$ | | | SI; HPV16 |
| 28 | TS00164406 3C | - | - | - | - | Negative |
| 29 | TS00187207 1 HED1D2 | $+^{\varepsilon}$ | | | | SI; HPV6 |
| 30 | TS00213305 1B | - | - | - | - | Negative |
| 31 | TS00232207 1 | - | - | - | - | Negative |
| 32 | TS00267305 3E | $+^{\delta}$ | | | | SI; HPV6 |
| 33 | TS00315505 1G | $+^{\delta}$ | | | | SI; HPV6 |
| 34 | TS00338107 2G | - | - | - | - | Negative |
| 35 | TS00486606 1A | - | - | - | - | Negative |
| 36 | TS00507307 2B | - | - | - | - | Negative |
| 37 | TS00624606 1 | | | | $+^{\delta,\varepsilon}$ | SI; HPV35 |
| 38 | TS00629005 1 | - | - | - | - | Negative |
| 39 | TS00727706 1 | - | - | - | - | Negative |
| 40 | TS00854605 3M | - | - | - | - | Negative |
| 41 | TS00858906 1C | - | - | - | - | Negative |
| 42 | TS00965006 3C | - | - | - | - | Negative |
| 43 | TS01129306 1B | $+^{\delta}$ | | | | SI; HPV6 |
| 44 | TS01218406 1 | - | - | - | - | Negative |
| 45 | TS01292806 2K | $+^{\delta}$ | | | | SI; HPV6 |
| 46 | TS01298705 1 | $+^{\delta,\varepsilon}$ | | | | SI; HPV6 |
| 47 | TS01353006 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 48 | TS01371006 1A | $+^{\delta}$ | | | | SI; HPV6 |
| 49 | TS01388406 4F | $+^{\delta,\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | DI; HPV6, α-HPV untypable |
| 50 | TS01390006 1E | $+^{\delta,\varepsilon}$ | | | | SI; HPV6 |
| 51 | TS01399006 1 | $+^{\varepsilon}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 52 | TS01423506 3H | $+^{\delta}$ | | | | SI; HPV6 |
| 53 | TS01514806 3D | $+^{\delta}$ | | | | SI; HPV6 |
| 54 | TS01596705 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 55 | TS01898205 1 | $+^{\delta}$ | | | | SI; HPV6 |
| 56 | TS01958205 1 | $+^{\delta}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 57 | TS02131305 2D | $+^{\delta}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 58 | TS02184105 2L | $+^{\delta}$ | $+^{\varepsilon}$ | | | DI; HPV6, HPV16 |
| 59 | TS02338805 1 | - | - | - | - | Negative |
| 60 | TS02518405 1 | $+^{\delta}$ | | | | SI; HPV6 |
| | Positive: | 34 | 7 | 1 | 1 | 36 |
| | Percentage: | 56.7 | 11.7 | 1.7 | 1.7 | 60.0 |
| | Total: | 60 | 60 | 60 | 60 | 60 |

Note:

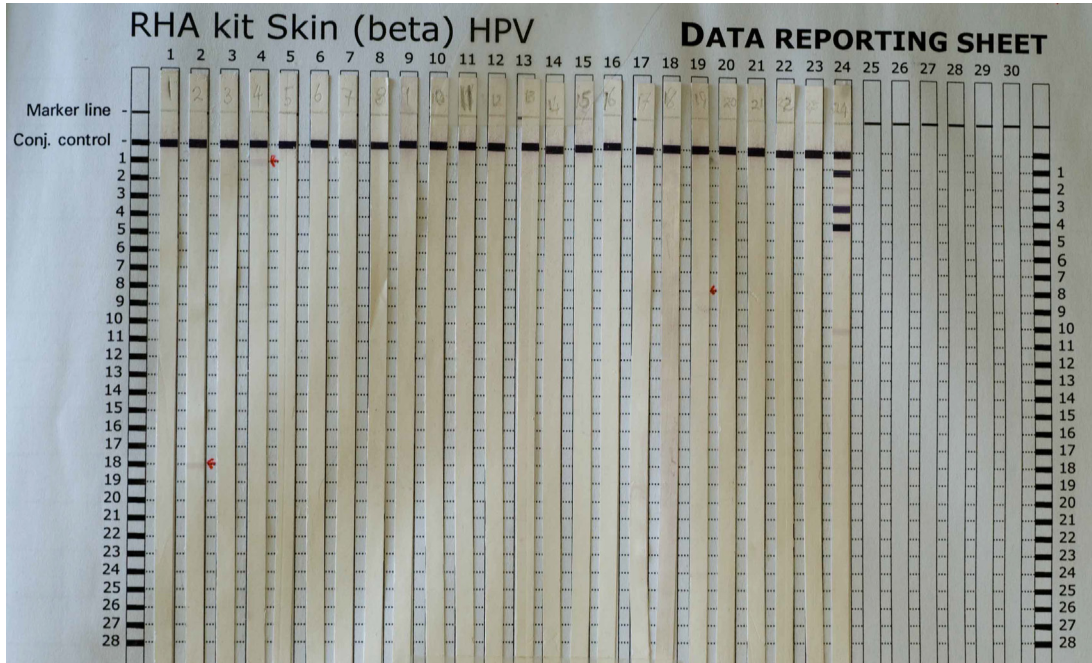
SI – single infection; DI – double infections; + positive; (+) positive for α-HPV untypable; - negative

$^{\delta}$ – Positive results obtained by using PCR and automated DNA sequencing methods

$^{\varepsilon}$ – Positive results obtained by using $SPF_{10}$-$LiPA_{25}$ method

# Appendix 24

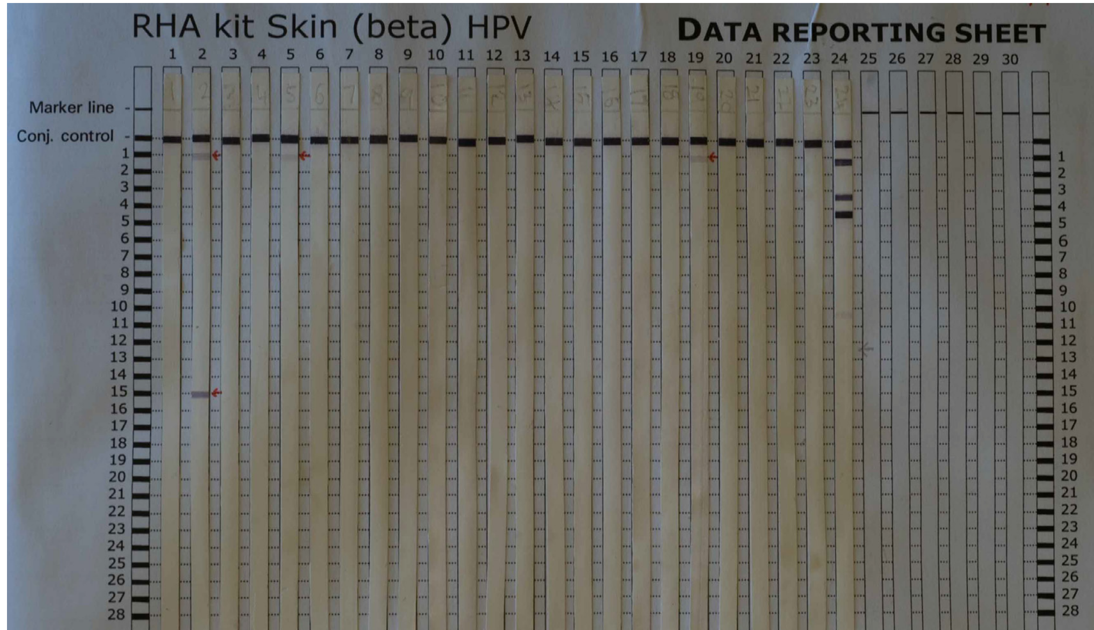
## Raw data - Beta HPV genotyping




| Strip | Sample ID | Reactive probes | Genotyping results | Remark |
|---|---|---|---|---|
| 1 | 1 (Tube #1) | | | |
| 2 | 2 (Tube #3) | 18 | HPV36 | |
| 3 | 3 (Tube #5) | | | |
| 4 | 4 (Tube #7) | 1 | β-HPV untypable | |
| 5 | 5 (Tube #9) | | | |
| 6 | 6 (Tube #11) | | | |
| 7 | 7 (Tube #13) | | | |
| 8 | 8 (Tube #15) | | | |
| 9 | 9 (Tube #17) | | | |
| 10 | 10 (Tube #20) | | | |
| 11 | 11 (Tube #21) | | | |
| 12 | 12 (Tube #23) | | | |
| 13 | 13 (Tube #25) | | | |
| 14 | 14 (Tube #27) | | | |
| 15 | 15 (Tube #29) | | | |
| 16 | 16 (Tube #31) | | | |
| 17 | 17 (Tube #33) | | | |
| 18 | 18 (Tube #35) | | | |
| 19 | 19 (Tube #37) | 8 | HPV15 | |
| 20 | 20 (Tube #39) | | | |
| 21 | Paraffin | | | |
| 22 | Reagent | | | |
| 23 | Water | | | |
| 24 | Control (HPV8) | 1,2,3,4,10,12 | 5,8,19 | |

**Appendix 24 (Continued)**

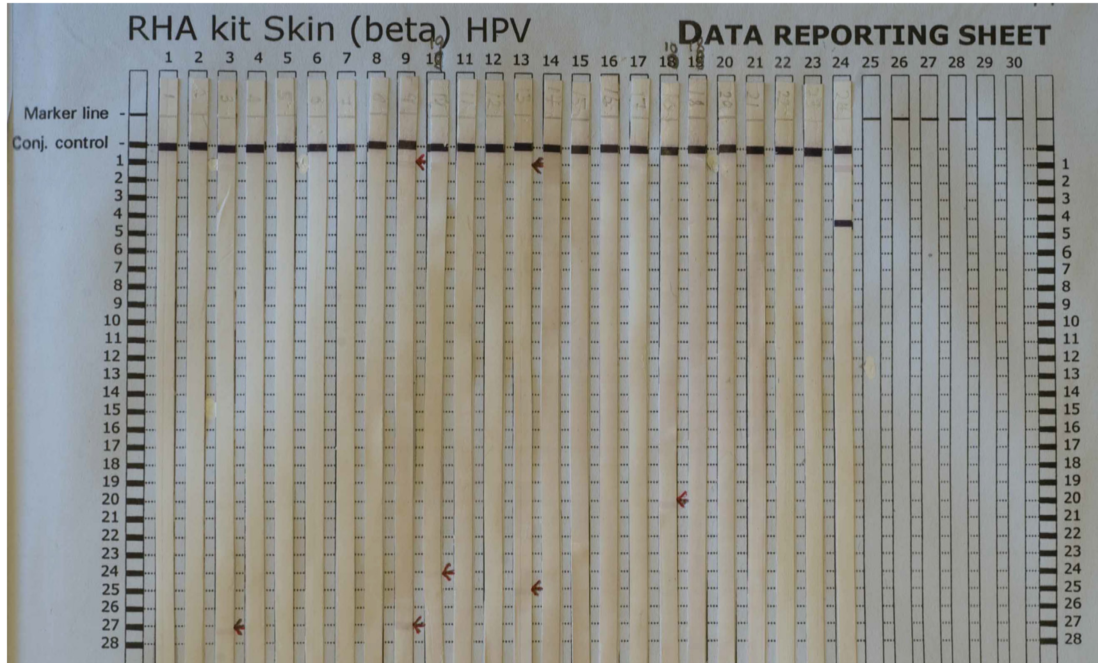
Raw data - Beta HPV genotyping




| Strip | Sample ID | Reactive probes | Genotyping results | Remark |
|---|---|---|---|---|
| 1 | 21 (Tube #41) | | | |
| 2 | 22 (Tube #43) | 1,15 | HPV23 | |
| 3 | 23 (Tube #45) | | | |
| 4 | 24 (Tube #47) | | | |
| 5 | 25 (Tube #49) | 1 | β-HPV untypable | |
| 6 | 26 (Tube #51) | | | |
| 7 | 27 (Tube #53) | | | |
| 8 | 28 (Tube #55) | | | |
| 9 | 29 (Tube #57) | | | |
| 10 | 30 (Tube #59) | | | |
| 11 | 31 (Tube #61) | | | |
| 12 | 32 (Tube #63) | | | |
| 13 | 33 (Tube #65) | | | |
| 14 | 34 (Tube #67) | | | |
| 15 | 35 (Tube #69) | | | |
| 16 | 36 (Tube #71) | | | |
| 17 | 37 (Tube #73) | | | |
| 18 | 38 (Tube #75) | | | |
| 19 | 39 (Tube #77) | 1 | β-HPV untypable | |
| 20 | 40 (Tube #79) | | | |
| 21 | Paraffin | | | |
| 22 | Reagent | | | |
| 23 | Water | | | |
| 24 | Control (HPV8) | 1,3,4,10,12 | 5,8,19 | |

**Appendix 24 (Continued)**

Raw data - Beta HPV genotyping




| Strip | Sample ID | Reactive probes | Genotyping results | Remark |
|---|---|---|---|---|
| 1 | 41 (Tube #81) | | | |
| 2 | 42 (Tube #83) | | | |
| 3 | 43 (Tube #85) | 27 | HPV93 | |
| 4 | 44 (Tube #87) | | | |
| 5 | 45 (Tube #89) | | | |
| 6 | 46 (Tube #91) | | | |
| 7 | 47 (Tube #93) | | | |
| 8 | 48 (Tube #95) | | | |
| 9 | 49 (Tube #97) | 1,27 | HPV93 | |
| 10 | 50 (Tube #99) | 20 | HPV38 | |
| 11 | 51 (Tube #101) | | | |
| 12 | 52 (Tube #103) | | | |
| 13 | 53 (Tube #105) | 1,25 | HPV80 | |
| 14 | 54 (Tube #107) | | | |
| 15 | 55 (Tube #109) | | | |
| 16 | 56 (Tube #111) | | | |
| 17 | 57 (Tube #114) | | | |
| 18 | 58 (Tube #115) | | | |
| 19 | 59 (Tube #117) | 24 | HPV76 | |
| 20 | 60 (Tube #119) | | | |
| 21 | Paraffin | | | |
| 22 | Reagent | | | |
| 23 | Water | | | |
| 24 | Control (HPV8) | 1,2,4 | 5,8 | |

# Appendix 25

## DIASSAY Skin (beta) HPV genotyping strip

### Genotyping Interpretation sheet

| Probe line | | HPV5 | HPV8 | | HPV9 | HPV12 | HPV14 | HPV15 | HPV17 | HPV19 | HPV20 | HPV21 | HPV22 | HPV23 | HPV24 | HPV25 | HPV36 | HPV37 | HPV38 | HPV47 | HPV49 | HPV75 | HPV76 | HPV80 | HPV92 | HPV93 | HPV96 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Uni | ( + ) | ( + ) | + | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) | ( + ) |
| 2 | HPV 5 | + | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | HPV 8 i | | ( + ) | + | | | ( + ) | | | | | | | | | | | | | | | | | | | | |
| 4 | HPV 8 ii | | + | + | | | | | | | | | | | | | | | | ( + ) | | | | | | | |
| 5 | HPV 9 | | | | + | | | | | | | | | | | | | | | | | | | | | | |
| 6 | HPV 12 | | | | | + | | | | | | | | | | | | | | | | | | | | | |
| 7 | HPV 14 | | | | | | + | | | | | | | | | | | | | | | | | | | | |
| 8 | HPV 15 | | | | | | | + | | | | | | | | | | | | | | | | | | | |
| 9 | HPV 17 | | | | | | | | + | | | | | | | | | | | | | | | | | | |
| 10 | HPV 19 | | | | | | | | | + | | | | | | | | | | | | | | | | | |
| 11 | HPV 20 | | | | | | | | | | + | | | | | | | | | | | | | | | | |
| 12 | HPV 21 | | | ( + ) | | | ( + ) | | | | | + | | | | | | | | | | | | | | | |
| 13 | cHPV 21 | | | | | | | | | | ( + ) | + | ( + ) | | | | | | | | | | | | | | |
| 14 | HPV 22 | | | | | | | | | | | | + | | | | | | | | | | | | | | |
| 15 | HPV 23 | | | | | | | | | | | | | + | | | | | | | | | | | | | |
| 16 | HPV 24 | | | | | | | | | | | | | | + | | | | | | | | | | | | |
| 17 | HPV 25 | | | | | | | | | | | | | | | + | | | | | | | | | | | |
| 18 | HPV 36 | | | | | | | | | | | | | | | | + | | | | | | | | | | |
| 19 | HPV 37 | | | | | | | | | | | | | | | | | + | | | | | | | | | |
| 20 | HPV 38 | | | | | | | | | | | | | | | | | | + | | | | | | | | |
| 21 | HPV 47 | | ( - ) | | | | | | | | | | | | | | | | | + | | | | | | | |
| 22 | HPV 49 | | | | | | | | | | | | | | | | | | | | + | | | | | | |
| 23 | HPV 75 | | | | | | | | | | | | | | | | | | | | | + | | | | | |
| 24 | HPV 76 | | | | | | | | | | | | | | | | | | | | | | + | | | | |
| 25 | HPV 80 | | | | | | | | | | | | | | | | | | | | | | | + | | | |
| 26 | HPV 92 | | | | | | | | | | | | | | | | | | | | | | | | + | | |
| 27 | HPV 93 | | | | | | | | | | | | | | | | | | | | | | | | | + | |
| 28 | HPV 96 | | | | | | | | | | | | | | | | | | | | | | | | | | + |

- The c HPV 21 probe is only used for type interpretation when the probeline above is positive
- HPV type 8 is recognized by probe lines 3 and 4, or, in absence of probe line 21, only by probe line 4
- In a mixed infection with HPV types 14 and 47 the presence of HPV type 8 cannot be established
- In a mixed infection with a combination of HPV types 8 or 14 on the one hand and HPV types 20 or 22 on the other, the presence of HPV type 21 cannot be established
- ( + ): Sometimes appears; not required for the determination of a genotype
- ( - ): Required to be negative

Note:

Reproduced with written permission from DDL, Voorburg, The Netherlands

# Appendix 26

## Beta HPV types detected by PM-PCR and RHA methods in pilot study samples

| Sample no. | Pathology code | β-HPV type | β-HPV status |
|---|---|---|---|
| 1 | 01209199 2 | - | Negative |
| 2 | 954593 B | HPV36 | SI; HPV36 |
| 3 | NS00196904 1 | - | Negative |
| 4 | NS00442204 1L | (+) | SI; β-HPV untypable |
| 5 | NS00642004 1 | - | Negative |
| 6 | NS00671203 1 | - | Negative |
| 7 | NS00711302 1B | - | Negative |
| 8 | NS00848002 3D | - | Negative |
| 9 | NS01/68103 1A | - | Negative |
| 10 | NS01/68103 1B | - | Negative |
| 11 | NS01020203 3K | - | Negative |
| 12 | NS01029003 1 | - | Negative |
| 13 | NS01076404 1B | - | Negative |
| 14 | NS01258903 1C | - | Negative |
| 15 | NS01291204 1C | - | Negative |
| 16 | NS01404702 1B | - | Negative |
| 17 | NS01643002 1A | - | Negative |
| 18 | NS01803103 1 | - | Negative |
| 19 | NS01864704 1B | HPV15 | SI; HPV15 |
| 20 | NS01960104 1 | - | Negative |
| 21 | NS02060804 1 | - | Negative |
| 22 | TS00012706 1 | HPV23 | SI; HPV23 |
| 23 | TS00044505 1 | - | Negative |
| 24 | TS00055407 1 | - | Negative |
| 25 | TS00059407 1 | (+) | SI; β-HPV untypable |
| 26 | TS00109407 2F | - | Negative |
| 27 | TS00148106 1 | - | Negative |
| 28 | TS00164406 3C | - | Negative |
| 29 | TS00187207 1 HED1D2 | - | Negative |
| 30 | TS00213305 1B | - | Negative |
| 31 | TS00232207 1 | - | Negative |
| 32 | TS00267305 3E | - | Negative |
| 33 | TS00315505 1G | - | Negative |
| 34 | TS00338107 2G | - | Negative |
| 35 | TS00486606 1A | - | Negative |
| 36 | TS00507307 2B | - | Negative |
| 37 | TS00624606 1 | - | Negative |
| 38 | TS00629005 1 | - | Negative |
| 39 | TS00727706 1 | (+) | SI; β-HPV untypable |
| 40 | TS00854605 3M | - | Negative |
| 41 | TS00858906 1C | - | Negative |
| 42 | TS00965006 3C | - | Negative |
| 43 | TS01129306 1B | HPV93 | SI; HPV93 |
| 44 | TS01218406 1 | - | Negative |
| 45 | TS01292806 2K | - | Negative |
| 46 | TS01298705 1 | - | Negative |
| 47 | TS01353006 1 | - | Negative |
| 48 | TS01371006 1A | - | Negative |
| 49 | TS01388406 4F | HPV93 | SI; HPV93 |
| 50 | TS01390006 1E | HPV38 | SI; HPV38 |
| 51 | TS01399006 1 | - | Negative |
| 52 | TS01423506 3H | - | Negative |
| 53 | TS01514806 3D | HPV80 | SI; HPV80 |
| 54 | TS01596705 1 | - | Negative |
| 55 | TS01898205 1 | - | Negative |
| 56 | TS01958205 1 | - | Negative |
| 57 | TS02131305 2D | - | Negative |
| 58 | TS02184105 2L | - | Negative |
| 59 | TS02338805 1 | HPV76 | SI; HPV76 |
| 60 | TS02518405 1 | - | Negative |
| | Positive: | 11 | 11 |
| | Percentage: | 18.3 | 18.3 |
| | Total: | 60 | 60 |

Note:
SI – single infection; (+) positive, β-HPV untypable; - negative

# Appendix 27

## Overall α- and β-HPV types detected by several methods in pilot study samples

| Sample no. | Pathology code | LR α-HPV | HR α-HPV | | | β-HPV | α- and β-HPV status |
|---|---|---|---|---|---|---|---|
| | | HPV6 | HPV16 | HPV18 | HPV35 | | |
| 1 | 01209199 2 | $+^{\delta}$ | | $+^{\varepsilon}$ | | - | DI; HPV6, HPV18 |
| 2 | 954593 B | $+^{\delta,\varepsilon}$ | | | | HPV36 | DI; HPV6; HPV36 |
| 3 | NS00196904 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 4 | NS00442204 1L | $+^{\delta}$ | $+^{\varepsilon}$ | | | (+) | MI; HPV6, HPV16; β-HPV untypable |
| 5 | NS00642004 1 | - | - | - | - | - | Negative |
| 6 | NS00671203 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 7 | NS00711302 1B | $+^{\delta}$ | | | | - | SI; HPV6 |
| 8 | NS00848002 3D | $+^{\delta}$ | | | | - | SI; HPV6 |
| 9 | NS01/68103 1A | $+^{\delta}$ | | | | - | SI; HPV6 |
| 10 | NS01/68103 1B | $+^{\delta}$ | $+^{\varepsilon}$ | | | - | DI; HPV6, HPV16 |
| 11 | NS01020203 3K | $+^{\delta,\varepsilon}$ | | | | - | SI; HPV6 |
| 12 | NS01029003 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 13 | NS01076404 1B | $+^{\delta}$ | | | | - | SI; HPV6 |
| 14 | NS01258903 1C | - | - | - | - | - | Negative |
| 15 | NS01291204 1C | $+^{\delta}$ | | | | - | SI; HPV6 |
| 16 | NS01404702 1B | $+^{\delta,\varepsilon}$ | | | | - | SI; HPV6 |
| 17 | NS01643002 1A | - | - | - | - | - | Negative |
| 18 | NS01803103 1 | - | - | - | - | - | Negative |
| 19 | NS01864704 1B | - | - | - | - | HPV15 | SI: HPV15 |
| 20 | NS01960104 1 | - | - | - | - | - | Negative |
| 21 | NS02060804 1 | - | - | - | - | - | Negative |
| 22 | TS00012706 1 | $+^{\delta,\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | HPV23 | MI; HPV6; α-HPV untypable; HPV23 |
| 23 | TS00044505 1 | - | - | - | - | - | Negative |
| 24 | TS00055407 1 | - | - | - | - | - | Negative |
| 25 | TS00059407 1 | - | - | - | - | (+) | SI; β-HPV untypable |
| 26 | TS00109407 2F | - | - | - | - | - | Negative |
| 27 | TS00148106 1 | | $+^{\delta}$ | | | - | SI; HPV16 |
| 28 | TS00164406 3C | - | - | - | - | - | Negative |
| 29 | TS00187207 1 HED1D2 | $+^{\varepsilon}$ | | | | - | SI; HPV6 |
| 30 | TS00213305 1B | - | - | - | - | - | Negative |
| 31 | TS00232207 1 | - | - | - | - | - | Negative |
| 32 | TS00267305 3E | $+^{\delta}$ | | | | - | SI; HPV6 |
| 33 | TS00315505 1G | $+^{\delta}$ | | | | - | SI; HPV6 |
| 34 | TS00338107 2G | - | - | - | - | - | Negative |
| 35 | TS00486606 1A | - | - | - | - | - | Negative |
| 36 | TS00507307 2B | - | - | - | - | - | Negative |
| 37 | TS00624606 1 | | | | $+^{\delta,\varepsilon}$ | - | SI; HPV35 |
| 38 | TS00629005 1 | - | - | - | - | - | Negative |
| 39 | TS00727706 1 | - | - | - | - | (+) | SI; β-HPV untypable |
| 40 | TS00854605 3M | - | - | - | - | - | Negative |
| 41 | TS00858906 1C | - | - | - | - | - | Negative |
| 42 | TS00965006 3C | - | - | - | - | - | Negative |
| 43 | TS01129306 1B | $+^{\delta}$ | | | | HPV93 | DI; HPV6; HPV93 |
| 44 | TS01218406 1 | - | - | - | - | - | Negative |
| 45 | TS01292806 2K | $+^{\delta}$ | | | | - | SI; HPV6 |
| 46 | TS01298705 1 | $+^{\delta,\varepsilon}$ | | | | - | SI; HPV6 |
| 47 | TS01353006 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 48 | TS01371006 1A | $+^{\delta}$ | | | | - | SI; HPV6 |
| 49 | TS01388406 4F | $+^{\delta,\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | $(+)^{\varepsilon}$ | HPV93 | MI; HPV6, α-HPV untypable; HPV93 |
| 50 | TS01390006 1E | $+^{\delta,\varepsilon}$ | | | | HPV38 | DI; HPV6; HPV38 |
| 51 | TS01399006 1 | $+^{\varepsilon}$ | $+^{\varepsilon}$ | | | - | DI; HPV6, HPV16 |
| 52 | TS01423506 3H | $+^{\delta}$ | | | | - | SI; HPV6 |
| 53 | TS01514806 3D | $+^{\delta}$ | | | | HPV80 | DI; HPV6; HPV80 |
| 54 | TS01596705 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 55 | TS01898205 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| 56 | TS01958205 1 | $+^{\delta}$ | $+^{\varepsilon}$ | | | - | DI; HPV6, HPV16 |
| 57 | TS02131305 2D | $+^{\delta}$ | $+^{\varepsilon}$ | | | - | DI; HPV6, HPV16 |
| 58 | TS02184105 2L | $+^{\delta}$ | $+^{\varepsilon}$ | | | - | DI; HPV6, HPV16 |
| 59 | TS02338805 1 | - | - | - | - | HPV76 | SI; HPV76 |
| 60 | TS02518405 1 | $+^{\delta}$ | | | | - | SI; HPV6 |
| | Positive: | 34 | 7 | 1 | 1 | 11 | 40 |
| | Percentage: | 56.7 | 11.7 | 1.7 | 1.7 | 18.3 | 66.7 |
| | Total: | 60 | 60 | 60 | 60 | 60 | 60 |

Note:

SI – single infection; DI – double infections; + positive; (+) positive but α-or β-HPV untypable (not counted in total); - negative

$^{\delta}$ – Positive results obtained by using PCR and automated DNA sequencing methods

$^{\varepsilon}$ – Positive results obtained by using $SPF_{10}$-$LiPA_{25}$ method

# Appendix 28

## The association of tobacco smoking and alcohol consumption with HPV status

| No. | Pathology code | Gender$^{\gamma}$ | Age | Age group$^{\delta}$ | Tobacco smoking and alcohol consumption status$^{\varepsilon}$ | HPV status$^{\S}$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | α-HPV | | β-HPV | Overall | Type of infection$^{\eta}$ |
| | | | | | | LR-HPV | HR-HPV | | | |
| 1 | 01209199 2 | M | 64 | 1 | 1 | HPV6 | HPV18 | - | + | DI |
| 2 | 954593 B | F | 68 | 2 | 0 | HPV6 | - | HPV36 | + | DI |
| 3 | NS00196904 1 | F | 97 | 2 | 0 | HPV6 | - | - | + | SI |
| 4 | NS00442204 1L | F | 97 | 2 | 0 | HPV6 | HPV16 | (+) | + | MI |
| 5 | NS00642004 1 | M | 78 | 2 | 3 | - | - | - | - | - |
| 6 | NS00671203 1 | M | 69 | 2 | 3 | HPV6 | - | - | + | SI |
| 7 | NS00711302 1B | F | 55 | 1 | 2 | HPV6 | - | - | + | SI |
| 8 | NS00848002 3D | F | 55 | 1 | 2 | HPV6 | - | - | + | SI |
| 9 | NS01/68103 1A | M | 74 | 2 | 3 | HPV6 | - | - | + | SI |
| 10 | NS01/68103 1B | M | 74 | 2 | 3 | HPV6 | HPV16 | - | + | DI |
| 11 | NS01020203 3K | M | 62 | 1 | 2 | HPV6 | - | - | + | SI |
| 12 | NS01029003 1 | M | 64 | 1 | 3 | HPV6 | - | - | + | SI |
| 13 | NS01076404 1B | M | 67 | 2 | 3 | HPV6 | - | - | + | SI |
| 14 | NS01258903 1C | M | 64 | 1 | 3 | - | - | - | - | - |
| 15 | NS01291204 1C | M | 77 | 2 | 2 | HPV6 | - | - | + | SI |
| 16 | NS01404702 1B | M | 60 | 1 | N/I | HPV6 | - | - | + | SI |
| 17 | NS01643002 1A | M | 60 | 1 | N/I | - | - | - | - | - |
| 18 | NS01803103 1 | M | 67 | 2 | 3 | - | - | - | - | - |
| 19 | NS01864704 1B | F | 78 | 2 | 3 | - | - | HPV15 | + | SI |
| 20 | NS01960104 1 | M | 66 | 2 | 3 | - | - | - | - | - |
| 21 | NS02060804 1 | F | 66 | 2 | 2 | - | - | - | - | - |
| 22 | TS00012706 1 | M | 65 | 1 | 3 | HPV6 | (+) | HPV23 | + | MI |
| 23 | TS00044505 1 | M | 52 | 1 | 2 | - | - | - | - | - |
| 24 | TS00055407 1 | M | 75 | 2 | 3 | - | - | - | - | - |
| 25 | TS00059407 1 | M | 55 | 1 | 3 | - | - | (+) | + | SI |
| 26 | TS00109407 2F | F | 59 | 1 | 3 | - | - | - | - | - |
| 27 | TS00148106 1 | M | 59 | 1 | 3 | | HPV16 | - | + | SI |
| 28 | TS00164406 3C | M | 65 | 1 | 3 | - | - | - | - | - |
| 29 | TS00187207 1 HED1D2 | F | 65 | 1 | 3 | HPV6 | - | - | + | SI |
| 30 | TS00213305 1B | F | 62 | 1 | 3 | - | - | - | - | - |
| 31 | TS00232207 1 | F | 63 | 1 | 3 | - | - | - | - | - |
| 32 | TS00267305 3E | M | 66 | 2 | 3 | HPV6 | - | - | + | SI |
| 33 | TS00315505 1G | M | 52 | 1 | 2 | HPV6 | - | - | + | SI |
| 34 | TS00338107 2G | M | 75 | 2 | 3 | - | - | - | - | - |
| 35 | TS00486606 1A | M | 36 | 1 | 3 | - | - | - | - | - |
| 36 | TS00507307 2B | F | 63 | 1 | 3 | - | - | - | - | - |
| 37 | TS00624606 1 | M | 62 | 1 | 2 | | HPV35 | - | + | SI |
| 38 | TS00629005 1 | F | 46 | 1 | 3 | - | - | - | - | - |
| 39 | TS00727706 1 | M | 67 | 2 | 3 | - | - | (+) | + | SI |
| 40 | TS00854605 3M | F | 46 | 1 | 3 | - | - | - | - | - |
| 41 | TS00858906 1C | M | 67 | 2 | 3 | - | - | - | - | - |
| 42 | TS00965006 3C | F | 66 | 2 | 1 | - | - | - | - | - |
| 43 | TS01129306 1B | F | 81 | 2 | 3 | HPV6 | - | HPV93 | + | DI |
| 44 | TS01218406 1 | F | 62 | 1 | 2 | - | - | - | - | - |
| 45 | TS01292806 2K | M | 69 | 2 | 3 | HPV6 | - | - | + | SI |
| 46 | TS01298705 1 | M | 84 | 2 | 3 | HPV6 | - | - | + | SI |
| 47 | TS01353006 1 | F | 63 | 1 | 3 | HPV6 | - | - | + | SI |
| 48 | TS01371006 1A | F | 75 | 2 | 1 | HPV6 | - | - | + | SI |
| 49 | TS01388406 4F | F | 81 | 2 | 3 | HPV6 | (+) | HPV93 | + | MI |
| 50 | TS01390006 1E | F | 62 | 1 | 2 | HPV6 | - | HPV38 | + | DI |
| 51 | TS01399006 1 | F | 59 | 1 | 2 | HPV6 | HPV16 | - | + | DI |
| 52 | TS01423506 3H | F | 59 | 1 | 2 | HPV6 | - | - | + | SI |
| 53 | TS01514806 3D | F | 75 | 2 | 1 | HPV6 | - | HPV80 | + | DI |
| 54 | TS01596705 1 | F | 78 | 2 | 2 | HPV6 | - | - | + | SI |
| 55 | TS01898205 1 | M | 64 | 1 | 2 | HPV6 | - | - | + | SI |
| 56 | TS01958205 1 | F | 70 | 2 | 3 | HPV6 | HPV16 | - | + | DI |
| 57 | TS02131305 2D | F | 70 | 2 | 3 | HPV6 | HPV16 | - | + | DI |
| 58 | TS02184105 2L | M | 62 | 1 | 2 | HPV6 | HPV16 | - | + | DI |
| 59 | TS02338805 1 | M | 70 | 2 | 1 | - | - | HPV76 | + | SI |
| 60 | TS02518405 1 | M | 62 | 1 | 3 | HPV6 | - | - | + | SI |

Note:-
$^{\gamma}$ - Gender; M – Male; F - Female
$^{\delta}$ - Age group was divided into two based on median age, Group 1 (age 16-65y), Group 2 (age 66-97).
$^{\varepsilon}$ - Tobacco smoking and alcohol consumption: 0 (no smoking, no drinking), 1 (no smoking, drinking), 2 (smoking, no drinking) and 3 (smoking and drinking). Two data (#16 and #17 with shaded) were not included in the analysis due to smoking and alcohol consumption status were not available.
$^{\S}$ - + HPV positive; - HPV negative
$^{\eta}$ - The type of HPV infection: single infection (SI), double infection (DI) and multiple infection (MI).

## Appendix 29

Semi-quantitative assessment of p16 immunohistochemistry staining

| No | Pathology ID | Field | | | | | | | | | | | | | | | | | | | | I | S |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | | 10 | | | |
| | | I | P | I | P | I | P | I | P | I | P | I | P | I | P | I | P | I | P | I | P | | |
| 1 | 01209199 2 | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 2 | 954593 B | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 3 | NS00196904 1 | 1 | 2 | 2 | 4 | 2 | 4 | 2 | 4 | 2 | 4 | 2 | 3 | 2 | 4 | 1 | 3 | 1 | 2 | 1 | 3 | **2** | + |
| 4 | NS00442204 1L | 3 | 1 | 3 | 1 | 3 | 1 | 2 | 1 | 3 | 5 | 2 | 5 | 3 | 1 | 3 | 5 | 3 | 1 | 2 | 5 | **3** | + |
| 5 | NS00642004 1 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | **1** | + |
| 6 | NS00671203 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 4 | 1 | 2 | 2 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | **2** | + |
| 7 | NS00711302 1B | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 8 | NS00848002 3D | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 9 | NS01/68103 1A | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 10 | NS01/68103 1B | 1 | 4 | 1 | 1 | 1 | 3 | 3 | 5 | 3 | 7 | 2 | 4 | 3 | 3 | 3 | 5 | 2 | 2 | 3 | 5 | **2** | + |
| 11 | NS01020203 3K | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 12 | NS01029003 1 | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 13 | NS01076404 1B | 2 | 2 | 1 | 3 | 1 | 4 | 1 | 4 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 6 | **1** | + |
| 14 | NS01258903 1C | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 15 | NS01291204 1C | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 3 | 1 | 2 | 1 | 2 | 2 | 3 | 2 | 1 | 2 | 3 | 1 | 1 | **2** | + |
| 16 | NS01404702 1B | 1 | 3 | 1 | 1 | 1 | 1 | 2 | 3 | 2 | 4 | 1 | 3 | 1 | 3 | 1 | 4 | 1 | 4 | 1 | 3 | **1** | + |
| 17 | NS01643002 1A | 1 | 4 | 1 | 3 | 1 | 3 | 1 | 3 | 1 | 2 | 1 | 3 | 1 | 4 | 1 | 3 | 1 | 4 | 1 | 3 | **1** | + |
| 18 | NS01803103 1 | 1 | 1 | 1 | 3 | 1 | 3 | 1 | 2 | 1 | 3 | 1 | 4 | 2 | 5 | 1 | 2 | 2 | 5 | 1 | 5 | **1** | + |
| 19 | NS01864704 1B | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 20 | NS01960104 1 | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 21 | NS02060804 1 | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 22 | TS00012706 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | **2** | + |
| 23 | TS00044505 1 | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 24 | TS00055407 1 | 2 | 3 | 3 | 6 | 3 | 7 | 2 | 3 | 2 | 2 | 1 | 3 | 2 | 2 | 3 | 5 | 3 | 6 | 3 | 4 | **2** | + |
| 25 | TS00059407 1 | 3 | 4 | 2 | 4 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | 3 | 2 | 1 | **2** | + |
| 26 | TS00109407 2F | 1 | 1 | 1 | 2 | 2 | 6 | 1 | 1 | 1 | 3 | 2 | 7 | 2 | 4 | 2 | 7 | 2 | 6 | 1 | 6 | **2** | + |
| 27 | TS00148106 1 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 4 | 2 | 3 | 3 | 4 | 2 | 3 | 2 | 3 | 2 | 6 | 2 | 2 | **3** | + |
| 28 | TS00164406 3C | 3 | 3 | 2 | 2 | 2 | 4 | 2 | 3 | 2 | 3 | 3 | 4 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 1 | **2** | + |
| 29 | TS00187207 1 HED1D2 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 2 | 2 | **2** | + |
| 30 | TS00213305 1B | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | **1** | + |
| 31 | TS00232207 1 | 2 | 8 | 2 | 8 | 2 | 7 | 2 | 8 | 2 | 9 | 2 | 9 | 2 | 9 | 2 | 9 | 2 | 9 | 2 | 9 | **2** | + |
| 32 | TS00267305 3E | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 6 | 2 | 3 | 1 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | 2 | **2** | + |
| 33 | TS00315505 1G | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 2 | **1** | + |
| 34 | TS00338107 2G | 2 | 3 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 3 | 2 | 3 | **2** | + |
| 35 | TS00486606 1A | 1 | 7 | 1 | 6 | 2 | 1 | 1 | 5 | 1 | 4 | 1 | 3 | 1 | 4 | 1 | 2 | 1 | 2 | 1 | 2 | **1** | + |
| 36 | TS00507307 2B | 2 | 8 | 2 | 7 | 2 | 6 | 2 | 7 | 2 | 6 | 2 | 6 | 2 | 4 | 2 | 3 | 2 | 3 | 2 | 3 | **2** | + |
| 37 | TS00624606 1 | 1 | 7 | 1 | 6 | 1 | 6 | 1 | 6 | 1 | 8 | 1 | 8 | 1 | 9 | 1 | 8 | 1 | 8 | 1 | 7 | **1** | + |
| 38 | TS00629005 1 | 2 | 3 | 2 | 4 | 2 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | **2** | + |
| 39 | TS00727706 1 | 1 | 4 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | **1** | + |
| 40 | TS00854605 3M | 1 | 2 | 1 | 2 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 3 | 1 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | **1** | + |
| 41 | TS00858906 1C | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | **1** | + |
| 42 | TS00965006 3C | 1 | 9 | 1 | 7 | 1 | 7 | 1 | 7 | 1 | 8 | 1 | 6 | 1 | 3 | 1 | 6 | 1 | 2 | 1 | 5 | **1** | + |
| 43 | TS01129306 1B | | | | | | | | | | | | | | | | | | | | | **Neg** | **Neg** |
| 44 | TS01218406 1 | 2 | 3 | 1 | 5 | 1 | 5 | 1 | 5 | 1 | 1 | 1 | 2 | 1 | 3 | 2 | 5 | 2 | 4 | 1 | 2 | **1** | + |
| 45 | TS01292806 2K | 1 | 1 | 1 | 1 | 2 | 5 | 2 | 5 | 2 | 5 | 1 | 5 | 1 | 1 | 1 | 5 | 1 | 1 | 1 | 1 | **1** | + |
| 46 | TS01298705 1 | 3 | 1 | 3 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 3 | 2 | 2 | 1 | 3 | 1 | **2** | + |
| 47 | TS01353006 1 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 3 | 2 | 3 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 3 | **2** | + |
| 48 | TS01371006 1A | 1 | 2 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 5 | 1 | 5 | 1 | 5 | 2 | 2 | 2 | 1 | 2 | 5 | **2** | + |
| 49 | TS01388406 4F | 1 | 4 | 1 | 3 | 1 | 1 | 1 | 3 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | **1** | + |
| 50 | TS01390006 1E | 1 | 5 | 1 | 5 | 1 | 1 | 1 | 5 | 2 | 1 | 2 | 1 | 1 | 5 | 1 | 1 | 1 | 5 | 1 | 1 | **1** | + |
| 51 | TS01399006 1 | 1 | 1 | 1 | 2 | 1 | 5 | 1 | 5 | 1 | 1 | 1 | 1 | 1 | 5 | 2 | 5 | 1 | 1 | 2 | 1 | **1** | + |
| 52 | TS01423506 3H | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 5 | 1 | 5 | 1 | 1 | 1 | 5 | 2 | 1 | 2 | 1 | 2 | 5 | **1** | + |
| 53 | TS01514806 3D | 1 | 8 | 1 | 5 | 1 | 5 | 1 | 2 | 2 | 3 | 1 | 4 | 1 | 3 | 1 | 1 | 1 | 3 | 1 | 2 | **1** | + |
| 54 | TS01596705 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | **1** | + |
| 55 | TS01898205 1 | 1 | 5 | 1 | 1 | 1 | 5 | 2 | 1 | 2 | 5 | 1 | 5 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | **1** | + |
| 56 | TS01958205 1 | 3 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 3 | 4 | 3 | 2 | 3 | 1 | **3** | + |
| 57 | TS02131305 2D | 1 | 1 | 1 | 1 | 2 | 3 | 2 | 2 | 1 | 2 | 1 | 2 | 2 | 2 | 1 | 1 | 2 | 2 | 1 | 1 | **1** | + |
| 58 | TS02184105 2L | 1 | 2 | 2 | 2 | 2 | 3 | 1 | 3 | 2 | 2 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 1 | 2 | **2** | + |
| 59 | TS02338805 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | **2** | + |
| 60 | TS02518405 1 | 2 | 2 | 2 | 2 | 3 | 4 | 2 | 4 | 2 | 3 | 3 | 3 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 2 | **2** | + |

Abbreviations:
I – Staining intensity; IC – intensity category (Neg=negative staining, 1 = 1+, 2 = 2+, 3 = 3+);
P – Percentage of positive staining (1=10%, 2=20%,…. 10=100%);
S – p16 status; Neg -negative, + positive

# Appendix 30

## (A) Clinical and histopathology parameters of 176 DNA samples for oral disease progression study

| No. | Plate | Pathology no. | Block | G$^{\alpha}$ | Age | Anatomic site | Code$^{\beta}$ | Pathology | Diff.$^{\gamma}$ | T$^{\delta}$ | N$^{\varepsilon}$ | Survival$^{\eta}$ | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1A | NS01006314 | 1 | M | 39 | BM | B | lichenoid reaction | | | | | |
| 2 | 1B | NS01017460 | 5J | M | 69 | Tongue | C | SCC | well | 2 | 0 | 60 | dead |
| 3 | 1C | NS01017677 | 2 | F | 49 | BM | B | lichenoid reaction | | | | | |
| 4 | 1D | NS01017679 | 1 | F | 45 | Tongue | B | Fep | | | | | |
| 5 | 1E | NS01017750 | 1 | M | 30 | Tongue | B | lichenoid reaction | | | | | |
| 6 | 1F | NS01017826 | 4D | F | 64 | Fom | C | SCC | mod | 4 | 2 | 8 | dead |
| 7 | 1G | NS01018047 | 1 | M | 50 | Tongue | B | lymphoid tissue | | | | | |
| 8 | 1H | NS01018047 | 2 | M | 50 | Tongue | B | lymphoid tissue | | | | | |
| 9 | 2B | NS01018532 | 1 | F | 73 | lower lip | B | Fep | | | | | |
| 10 | 2C | NS01018560 | 1 | F | 61 | BM | B | LP | | | | | |
| 11 | 2E | NS02000314 | 2 | M | 68 | BM | B | Fep | | | | | |
| 12 | 2F | NS02000316 | 7C | F | 66 | Fom | C | SCC | mod | 1 | 0 | 60 | alive |
| 13 | 2G | NS02000370 | 1 | M | 35 | Tongue | B | Fep | | | | | |
| 14 | 2H | NS02000373 | 1 | M | 54 | Gingivae | B | LP | | | | | |
| 15 | 3A | NS02000961 | 1 | F | 45 | Tongue | B | LP | | | | | |
| 16 | 3B | NS02001012 | 1A | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 17 | 3C | NS02001012 | 1B | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 18 | 3D | NS02001012 | 1C | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 19 | 3E | NS02001012 | 1D | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 20 | 3F | NS02001012 | 1E | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 21 | 3G | NS02001012 | 1F | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 22 | 3H | NS02001012 | 1G | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 23 | 4A | NS02001012 | 1H | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 24 | 4B | NS02001012 | 1I | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 25 | 4C | NS02001012 | 1J | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 26 | 4D | NS02001012 | 1K | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 27 | 4E | NS02001012 | 1L | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 28 | **4F** | NS02001012 | 1M | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 29 | 4G | NS02001012 | 1N | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 30 | 4H | NS02001012 | 1O | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 31 | 5A | NS02001012 | 1P | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 32 | 5B | NS02001012 | 2A | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 33 | 5C | NS02001012 | 2B | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 34 | 5D | NS02001012 | 2C | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 35 | 5E | NS02001012 | 2D | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 36 | 5F | NS02001012 | 2E | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 37 | 5G | NS02001012 | 2F | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 38 | 5H | NS02001012 | 2G | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 39 | 6A | NS02001012 | 2H | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 40 | 6B | NS02001012 | 2I | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 41 | 6C | NS02001012 | 2J | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 42 | 6D | NS02001012 | 2K | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 43 | 6E | NS02001012 | 2L | F | 45 | soft palate | C | SCC | mod | 1 | 0 | 60 | alive |
| 44 | 6G | NS02001225 | 1 | M | 46 | BM | B | lichenoid reaction | | | | | |
| 45 | 6H | NS01001286 | 1B | M | 32 | Skin | B | Inflame | | | | | |
| 46 | 7A | NS02001288 | 1 | M | 34 | Gingivae | B | LP | | | | | |
| 47 | 7B | NS02001290 | 1 | M | 49 | RMP | B | Fep | | | | | |
| 48 | 7C | NS02001293 | 1 | F | 46 | BM | B | Fep | | | | | |
| 49 | 7D | NS02001294 | 1 | F | 72 | BM | B | lichenoid reaction | | | | | |
| 50 | 7F | NS02001647 | 1 | M | 46 | BM | B | LP | | | | | |
| 51 | 7G | NS02001832 | 1 | M | 66 | Tongue | B | Fep | | | | | |
| 52 | 8A | NS02001962 | 1D | M | 60 | Fom | D | Cis | | | | | |
| 53 | 8B | NS02001967 | 1 | M | 32 | Tongue | B | lichenoid reaction | | | | | |
| 54 | 8C | NS02001970 | 1 | F | 52 | BM | B | LP | | | | | |
| 55 | 8D | NS02002097 | 1 | F | 79 | soft palate | C | SCC | basaloid | 2 | 0 | 13 | dead |
| 56 | 8E | NS02002098 | 1 | M | 63 | soft palate | C | SCC | mod | 2 | 0 | 20 | dead |
| 57 | 8F | NS02002098 | 2 | M | 63 | soft palate | C | SCC | mod | 2 | 0 | 20 | dead |
| 58 | 8G | NS02002185 | 1 | M | 59 | Fom | C | SCC | mod | 3 | 0 | 60 | alive |
| 59 | 8H | NS02002359 | 1 | F | 62 | BM | B | Fep | | | | | |

Note: $^{\alpha}$ - Gender, F – Female, M - Male; $^{\beta}$ – Pathological status, B – benign, D – dysplasia, C – carcinoma; Anatomic site, BM – buccal mucosa, Fom – Floor of the mouth, RMP – Retromolar pad, Pathology, SCC – squamous cell carcinoma, LP – lichen planus, Cis – carcinoma in situ, Fep – fibrioepithelioma of Pinkus; mod – moderate; $^{\gamma}$ – Cellular differentiation; mod – moderately differentiated, $^{\delta}$ – Tumour size; $^{\varepsilon}$ – Regional Lymph Nodes; $^{\eta}$ – Survival Time (months); Grey shaded samples, #56, #57, #73, #80 and #105 exhibited recurrence.

## Appendix 30 (Continued)

(A) Clinical and histopathology parameters of 176 DNA samples for oral disease progression study

| No. | Plate | Pathology no. | Block | G$^{\alpha}$ | Age | Anatomic site | Code$^{\beta}$ | Pathology | Diff.$^{\gamma}$ | T$^{\delta}$ | N$^{\varepsilon}$ | Survival$^{\eta}$ | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 60 | 9A | NS02002447 | 1 | F | 65 | hard palate | D | severe dysplasia | | | | | |
| 61 | 9B | NS02002905 | 1 | M | 48 | Tongue | B | LP | | | | | |
| 62 | 9C | NS02002906 | 1 | F | 64 | Fom | B | LP | | | | | |
| 63 | 9D | NS02003275 | 3J | M | 81 | Fom | C | SCC | basaloid | 1 | 0 | 60 | alive |
| 64 | 9E | NS02003306 | 1A | F | 57 | BM | B | Fep | | | | | |
| 65 | 9F | NS02003307 | 1B | F | 38 | BM | B | Fep | | | | | |
| 66 | 9G | NS02003409 | 1 | M | 35 | BM | B | Fep | | | | | |
| 67 | 9H | NS02003552 | 3A | M | 59 | Fom | C | SCC | mod | 2 | 0 | 60 | alive |
| 68 | 10A | NS02003610 | 1 | F | 47 | Fom | B | Fep | | | | | |
| 69 | 10B | NS02003612 | 1 | F | 88 | lower lip | B | Fep | | | | | |
| 70 | 10C | NS02003710 | 1 | F | 40 | upper lip | B | Fep | | | | | |
| 71 | 10D | NS02003712 | 1 | F | 61 | BM | B | LP | | | | | |
| 72 | 10E | NS02003914 | 1 | M | 57 | upper lip | B | Fep | | | | | |
| 73 | 10F | NS02004213 | 1 | F | 66 | Fom | C | SCC | mod | 2 | 0 | 60 | alive |
| 74 | 10G | NS02004435 | 1 | F | 64 | BM | B | LP | | | | | |
| 75 | 10H | NS02004442 | 3B | M | 63 | RMP | C | SCC | mod | 2 | 0 | 20 | dead |
| 76 | 11A | NS02004521 | 1 | F | 62 | BM | B | Fep | | | | | |
| 77 | 11C | NS02005157 | 1 | M | 84 | lower lip | C | SCC | mod | 1 | 0 | 60 | alive |
| 78 | 11D | NS02005229 | 3B | F | 66 | Fom | C | SCC | mod | 1 | 0 | 60 | alive |
| 79 | 11E | NS02005343 | 1 | F | 34 | BM | B | Fep | | | | | |
| 80 | 11F | NS02005525 | 1C | M | 75 | Tongue | C | SCC | mod | 2 | 0 | 19 | dead |
| 81 | 11G | NS02005565 | 1 | M | 53 | BM | B | lichenoid reaction | | | | | |
| 82 | 11H | NS02005568 | 1 | F | 59 | BM | B | Fep | | | | | |
| 83 | 12A | NS02005642 | 1B | M | 71 | Tongue | C | SCC | well | 2 | 0 | 2 | dead |
| 84 | 12B | NS02006000 | 1A | M | 66 | Tongue | B | Fep | | | | | |
| 85 | 12C | NS02006000 | 1B | M | 66 | Tongue | B | Fep | | | | | |
| 86 | 12E | NS02006074 | 1 | F | 34 | BM | B | Fep | | | | | |
| 87 | 12F | NS02006089 | 1 | M | 55 | Tongue | C | SCC | mod | 2 | 0 | 31 | dead |
| 88 | 13A | NS02006419 | 1A | M | 74 | Tongue | C | SCC | mod | 2 | 0 | 29 | dead |
| 89 | 13C | NS02006721 | 1B | M | 84 | lower lip | C | SCC | mod | 1 | 0 | 60 | alive |
| 90 | 13D | NS02006728 | 3 | F | 67 | Tongue | C | SCC | mod | 2 | 0 | 1 | dead |
| 91 | 13E | NS02006829 | 1 | F | 41 | BM | B | Fep | | | | | |
| 92 | 13F | NS02006965 | 1 | M | 35 | hard palate | B | LP | | | | | |
| 93 | 13G | NS02007068 | 3A | M | 55 | Tongue | C | SCC | mod | 2 | 1 | 31 | dead |
| 94 | 13H | NS02007111 | 1A | F | 78 | Fom | D | Cis | | | | | |
| 95 | 14A | NS02007112 | 1A | F | 86 | Tongue | C | SCC | mod | 3 | 0 | 4 | dead |
| 96 | 14B | NS02007115 | 1 | F | 57 | Tongue | B | Fep | | | | | |
| 97 | 14C | NS02007236 | 1C | M | 84 | lower lip | C | SCC | well | 1 | 0 | 40 | dead |
| 98 | 14D | NS02007455 | 1B | M | 16 | BM | B | Fep | | | | | |
| 99 | 14E | NS02007591 | 1A | F | 62 | Tongue | B | LP | | | | | |
| 100 | 14F | NS02007598 | 1 | F | 57 | Gingivae | B | LP | | | | | |
| 101 | 14G | NS02007665 | 1 | M | 65 | Gingivae | B | Fep | | | | | |
| 102 | 14H | NS02007740 | 1 | M | 49 | BM | D | severe dysplasia | | | | | |
| 103 | 15A | NS02007766 | 1 | F | 71 | Tongue | C | SCC | mod | 1 | 0 | 60 | alive |
| 104 | 15B | NS02007899 | 1 | F | 46 | Tongue | B | Fep | | | | | |
| 105 | 15C | NS02008161 | 1A | F | 71 | Tongue | C | SCC | mod | 1 | 0 | 60 | alive |
| 106 | 15D | NS02008655 | 1A | F | 57 | Gingivae | B | Inflame | | | | | |
| 107 | 15E | NS02008741 | 1 | F | 79 | BM | C | SCC | poor | 3 | 0 | 3 | dead |
| 108 | 15F | NS02008759 | 1 | M | 43 | soft palate | B | Fep | | | | | |
| 109 | 15G | NS02009289 | 1 | M | 85 | BM | C | SCC | mod | 2 | 0 | 3 | dead |
| 110 | 15H | NS02009377 | 1 | M | 65 | BM | B | Fep | | | | | |
| 111 | 16A | NS02009378 | 1 | F | 45 | Fom | B | Inflame | | | | | |
| 112 | 16B | NS02009385 | 1 | F | 59 | soft palate | B | LP | | | | | |
| 113 | 16C | NS02009890 | 1 | M | 53 | Tongue | B | LP | | | | | |
| 114 | 16D | NS02009892 | 1 | F | 45 | Tongue | B | LP | | | | | |
| 115 | 16E | NS02010172 | 1 | M | 37 | BM | B | LP | | | | | |
| 116 | 16F | NS02010172 | 2 | M | 75 | BM | B | Fep | | | | | |
| 117 | 16G | NS02010245 | 1 | M | 75 | BM | B | Fep | | | | | |
| 118 | 16H | NS02010301 | 1 | F | 23 | Gingivae | B | Fep | | | | | |
| 119 | 17A | NS02010352 | 1 | M | 90 | RMP | C | SCC | mod | 2 | 0 | 2 | dead |

## Appendix 30 (Continued)

(A) Clinical and histopathology parameters of 176 DNA samples for oral disease progression study

| No. | Plate | Pathology no. | Block | G$^{\alpha}$ | Age | Anatomic site | Code$^{\beta}$ | Pathology | Diff.$^{\gamma}$ | T$^{\delta}$ | N$^{\varepsilon}$ | Survival$^{\eta}$ | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 120 | 17B | NS02010409 | 1 | F | 61 | BM | B | Fep | | | | | |
| 121 | 17C | NS02010583 | 1 | F | 81 | soft palate | B | Fep | | | | | |
| 122 | 17D | NS02010642 | 1A | M | 54 | RMP | C | SCC | well | 3 | 0 | 60 | alive |
| 123 | 17E | NS02010835 | 1 | F | 60 | Tongue | B | Inflame | | | | | |
| 124 | 17F | NS02010904 | 1 | M | 28 | BM | B | LP | | | | | |
| 125 | 17G | NS02011108 | 1A | M | 91 | lower lip | C | SCC | well | 1 | 0 | 17 | dead |
| 126 | 17H | NS02011344 | 1 | M | 90 | Fom | C | SCC | well | 2 | 0 | 60 | alive |
| 127 | 18A | NS02011372 | 1A | M | 51 | Tongue | C | SCC | poor | 3 | 0 | 60 | alive |
| 128 | 18B | NS02011466 | 2A | F | 68 | Tongue | C | SCC | mod | 3 | 0 | 60 | dead |
| 129 | 18C | NS02011734 | 1 | M | 63 | soft palate | D | Cis | | | | | |
| 130 | 18D | NS02011761 | 1B | F | 73 | Tongue | C | SCC | poor | 3 | 0 | 39 | dead |
| 131 | 18E | NS02011779 | 1 | M | 56 | BM | B | LP | | | | | |
| 132 | 18F | NS02012088 | 1A | M | 63 | Tongue | B | LP | | | | | |
| 133 | 18G | NS02012156 | 1 | F | 99 | Tongue | C | SCC | well | 2 | 0 | 3 | dead |
| 134 | 18H | NS02012233 | 1 | M | 63 | Fom | D | mod dyplasia | | | | | |
| 135 | 19A | NS02012381 | 1 | F | 68 | BM | B | LP | | | | | |
| 136 | 19B | NS02012538 | 1A | M | 77 | lower lip | B | Inflame | | | | | |
| 137 | 19C | NS02012753 | 1 | M | 43 | BM | B | Fep | | | | | |
| 138 | 19D | NS02012797 | 1 | M | 54 | Fom | C | SCC | mod | 3 | 0 | 14 | dead |
| 139 | 19E | NS02012798 | 2 | M | 72 | soft palate | C | SCC | poor | 3 | 0 | 56 | dead |
| 140 | 19F | NS02013040 | 1 | F | 57 | BM | B | LP | | | | | |
| 141 | 19G | NS02013296 | 3C | M | 80 | Fom | C | SCC | mod | 4 | 2 | 60 | alive |
| 142 | 19H | NS02013597 | 1 | M | 63 | soft palate | D | severe dysplasia | | | | | |
| 143 | 20A | NS02013634 | 1B | F | 54 | Tongue | B | LP | | | | | |
| 144 | 20B | NS02013654 | 1 | M | 38 | BM | B | LP | | | | | |
| 145 | 20C | NS02013666 | 3O | M | 63 | Tongue | C | SCC | poor | 4 | 2 | 2 | dead |
| 146 | 20D | NS02013773 | 1 | F | 82 | BM | B | LP | | | | | |
| 147 | 20E | NS02013919 | 1 | F | 44 | Tongue | B | Fep | | | | | |
| 148 | 20F | NS02013948 | 1C | M | 69 | Tongue | C | SCC | well | 1 | 0 | 60 | alive |
| 149 | 20G | NS02014046 | 1D | M | 54 | Gingivae | C | SCC | mod | 2 | 1 | 13 | dead |
| 150 | 20H | NS02014101 | 1A | F | 60 | BM | C | SCC | mod | 2 | 0 | 60 | alive |
| 151 | 21A | NS02014375 | 1 | M | 50 | BM | B | Fep | | | | | |
| 152 | 21B | NS02014416 | 2B | F | 73 | Tongue | C | SCC | mod | 1 | 0 | 33 | dead |
| 153 | 21C | NS02014462 | 1 | F | 53 | lower lip | B | Fep | | | | | |
| 154 | 21D | NS02014490 | 1A | F | 71 | soft palate | C | SCC | mod | 4 | 2 | 54 | dead |
| 155 | 21E | NS02014538 | 1 | M | 30 | BM | B | LP | | | | | |
| 156 | 21F | NS02014854 | 1 | F | 65 | BM | B | Fep | | | | | |
| 157 | 21G | NS02014921 | 1 | M | 45 | Fom | D | mild dysplasia | | | | | |
| 158 | 21H | NS02015136 | 1 | M | 56 | Tongue | B | Inflame | | | | | |
| 159 | 22A | NS02015366 | 1B | F | 72 | Gingivae | C | SCC | mod | 1 | 2 | 8 | dead |
| 160 | 22B | NS02015903 | 1 | F | 64 | Fom | D | severe dysplasia | | | | | |
| 161 | 22C | NS02016061 | 1 | M | 57 | soft palate | C | SCC | poor | 3 | 0 | 13 | dead |
| 162 | 22D | NS02016362 | 2A | M | 78 | Fom | C | SCC | mod | 1 | 1 | 14 | dead |
| 163 | 22E | NS02016901 | 8D | F | 68 | Fom | D | Cis | | | | | |
| 164 | 22F | NS02017209 | 1 | F | 56 | BM | B | LP | | | | | |
| 165 | 22G | NS02017309 | 1A | F | 58 | BM | B | Fep | | | | | |
| 166 | 22H | NS02017374 | 1 | F | 67 | Fom | D | Cis | | | | | |
| 167 | 23A | NS02017375 | 2 | M | 71 | hard palate | B | LP | | | | | |
| 168 | 23B | NS02017558 | 1 | M | 97 | Fom | D | Cis | | | | | |
| 169 | 23C | NS02017642 | 5Q | F | 62 | Gingivae | C | SCC | well | 2 | 0 | 60 | dead |
| 170 | 23D | NS02017902 | 1 | M | 65 | Gingivae | C | SCC | poor | 3 | 1 | 8 | dead |
| 171 | 23E | NS02017946 | 2F | M | 57 | Fom | C | SCC | poor | 3 | 2 | 11 | dead |
| 172 | 23F | NS02018053 | 1 | F | 77 | Tongue | C | SCC | poor | 3 | 1 | 60 | alive |
| 173 | 23G | NS02018054 | 1 | F | 63 | BM | B | LP | | | | | |
| 174 | 23H | NS02018368 | 4F | F | 80 | BM | B | LP | | | | | |
| 175 | 24A | NS02018494 | 1A | F | 47 | Tongue | C | SCC | poor | 2 | 1 | 3 | dead |
| 176 | 24B | NS02018494 | 1B | F | 47 | Tongue | C | SCC | poor | 2 | 1 | 3 | dead |

## Appendix 30 (Continued)

### (B) Clinical and histopathology parameters of 7 DNA samples (normal tissues)

| No. | Plate | Pathology no. | Block | G[α] | Age | Anatomic site | Code[β] | Pathology | Status |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2D | NS02000126 | 1 | M | 77 | Tongue | N | Normal | Dead |
| 2 | 6F | NS02001175 | 1D | F | 64 | Tonsil | N | Normal | |
| 3 | 7E | NS02001437 | 1 | M | 68 | RMP | N | Normal | |
| 4 | 11B | NS02004522 | 1 | F | 52 | Tongue | N | Normal | |
| 5 | 12D | NS02006017 | 1 | F | 27 | BM | N | Normal | |
| 6 | 12G | NS02006394 | 1 | M | 46 | Tongue | N | Normal | |
| 7 | 12H | NS02006395 | 1 | M | 47 | lower lip | N | Normal | |

Note: [α] - Gender, F – Female, M - Male; [β] – Pathological status, N – normal; Anatomic site, BM – buccal mucosa, RMP – Retromolar pad.

### (C) DNA purity and DNA yield of 7 extracted DNA samples (normal tissues)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA | DNA yield | Dilution for PCR (10 ng/µl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | purity | (µg/µl) | DNA | $dH_2O$ | Total |
| 1 | 2D | NS02000126 | 1 | 4.83 | 5.13 | 4.72 | 5.09 | 4.70 | 5.01 | 0.936 | 0.238 | 8.4 | 191.6 | 200 |
| 2 | 6F | NS02001175 | 1D | 25.03 | 23.66 | 25.81 | 24.55 | 25.90 | 24.64 | 1.759 | 0.143 | 14.0 | 186.0 | 200 |
| 3 | 7E | NS02001437 | 1 | 18.29 | 17.48 | 18.60 | 17.78 | 18.75 | 17.90 | 1.768 | 0.095 | 21.0 | 179.0 | 200 |
| 4 | 11B | NS02004522 | 1 | 7.91 | 7.78 | 8.17 | 8.05 | 8.17 | 8.04 | 1.016 | 0.404 | 4.9 | 195.1 | 200 |
| 5 | 12D | NS02006017 | 1 | 2.51 | 2.75 | 2.47 | 2.71 | 2.41 | 2.68 | 0.908 | 0.123 | 16.2 | 183.8 | 200 |
| 6 | 12G | NS02006394 | 1 | 1.41 | 1.61 | 1.52 | 1.73 | 1.48 | 1.72 | 0.872 | 0.074 | 27.2 | 172.8 | 200 |
| 7 | 12H | NS02006395 | 1 | 3.91 | 4.14 | 4.07 | 4.29 | 4.09 | 4.33 | 0.946 | 0.201 | 9.9 | 190.1 | 200 |

### (D) PCR results for β-globin and L1 for α-HPV (normal tissues)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 1 | 2D | NS02000126 | 1 | + | - | + | + | + |
| 2 | 6F | NS02001175 | 1D | + | - | + | + | + |
| 3 | 7E | NS02001437 | 1 | + | - | - | + | + |
| 4 | 11B | NS02004522 | 1 | + | - | + | + | + |
| 5 | 12D | NS02006017 | 1 | + | - | - | - | - |
| 6 | 12G | NS02006394 | 1 | + | - | + | + | + |
| 7 | 12H | NS02006395 | 1 | + | - | - | - | - |
| | | PCR positive (%): | | 7 (100.0) | 0 (0.0) | 4 (57.1) | 5 (71.4) | 5 (71.4) |
| | | PCR negative (%): | | 0 (0.0) | 7 (100.0) | 3 (42.9) | 2 (28.6) | 2 (28.6) |
| | | Total samples: | | 7 | 7 | 7 | 7 | 7 |

Note: + Positive PCR and - Negative PCR

### (E) DNA sequencing and multiple sequences alignment results (normal tissues)

| No. | Plate | Pathology code | Block | Expected amplicon size (bp) | PCR purification methods[α] | HPV identification |
|---|---|---|---|---|---|---|
| 1 | 2D | NS02000126 | 1 | 190 | PCR | Negative |
| 2 | 6F | NS02001175 | 1D | 140 | PCR | Negative |
| 3 | 7E | NS02001437 | 1 | 140 | PCR | Negative |
| 4 | 11B | NS02004522 | 1 | 140 | Gel | HPV 16 |
| 5 | 12G | NS02006394 | 1 | 140 | PCR | Negative |

Notes:

[α] - PCR product purification methods: PCR - QIAquick PCR Purification Kit (Qiagen); Gel - QIAquick Gel Extraction Kit (Qiagen)

## Appendix 31

DNA purity and DNA yield of 84 extracted DNA samples (Benign)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA | DNA yield | Dilution for PCR (10 ng/µl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | purity | (µg/µl) | DNA | $dH_2O$ | Total |
| 1 | 1A | NS01006314 | 1 | 0.61 | 0.54 | 0.73 | 0.67 | 0.88 | 0.82 | 1.098 | 0.037 | 54.1 | 145.9 | 200 |
| 2 | 1C | NS01017677 | 2 | 7.62 | 8.08 | 5.88 | 6.38 | 5.42 | 5.90 | 0.928 | 0.315 | 6.3 | 193.7 | 200 |
| 3 | 1D | NS01017679 | 1 | 4.41 | 4.28 | 4.39 | 4.26 | 4.39 | 4.27 | 1.836 | 0.014 | 140.5 | 59.5 | 200 |
| 4 | 1E | NS01017750 | 1 | 12.97 | 12.64 | 13.05 | 12.77 | 12.92 | 12.65 | 2.456 | 0.025 | 80.0 | 120.0 | 200 |
| 5 | 1G | NS01018047 | 1 | 28.14 | 27.05 | 28.82 | 27.73 | 28.83 | 27.75 | 2.320 | 0.096 | 20.9 | 179.1 | 200 |
| 6 | 1H | NS01018047 | 2 | 5.15 | 4.96 | 5.15 | 4.96 | 5.12 | 4.92 | 2.044 | 0.019 | 106.2 | 93.8 | 200 |
| 7 | 2B | NS01018532 | 1 | 2.35 | 2.29 | 2.59 | 2.52 | 2.73 | 2.73 | 0.975 | 0.126 | 15.9 | 184.1 | 200 |
| 8 | 2C | NS01018560 | 1 | 2.92 | 2.93 | 3.2 | 3.24 | 3.81 | 3.62 | 1.006 | 0.167 | 12.0 | 188.0 | 200 |
| 9 | 2E | NS02000314 | 2 | 2.87 | 3.35 | 2.57 | 3.06 | - | - | 0.849 | 0.136 | 14.7 | 185.3 | 200 |
| 10 | 2G | NS02000370 | 1 | 23.83 | 23.46 | 24.37 | 24.07 | 24.38 | 24.05 | 1.753 | 0.039 | 51.5 | 148.5 | 200 |
| 11 | 2H | NS02000373 | 1 | 13.92 | 13.55 | 14.23 | 13.85 | 14.35 | 13.95 | 2.179 | 0.036 | 55.8 | 144.2 | 200 |
| 12 | 3A | NS02000961 | 1 | 12.38 | 12.12 | 12.64 | 12.34 | 12.62 | 12.34 | 2.460 | 0.024 | 84.5 | 115.5 | 200 |
| 13 | 6G | NS02001225 | 1 | 15.35 | 14.73 | 15.50 | 14.89 | 15.56 | 14.92 | 1.733 | 0.074 | 27.1 | 172.9 | 200 |
| 14 | 6H | NS01001286 | 1B | 15.24 | 14.65 | 15.44 | 14.86 | 15.50 | 14.93 | 1.784 | 0.066 | 30.3 | 169.7 | 200 |
| 15 | 7A | NS02001288 | 1 | 1.37 | 1.51 | 1.97 | 2.07 | 2.58 | 2.72 | 1.069 | 0.105 | 19.0 | 181.0 | 200 |
| 16 | 7B | NS02001290 | 1 | 15.48 | 15.02 | 15.71 | 15.25 | 15.76 | 15.26 | 1.652 | 0.060 | 33.3 | 166.7 | 200 |
| 17 | 7C | NS02001293 | 1 | 4.47 | 4.26 | 3.78 | 3.59 | 3.68 | 3.49 | 1.052 | 0.199 | 10.1 | 189.9 | 200 |
| 18 | 7D | NS02001294 | 1 | 14.12 | 13.61 | 14.36 | 13.81 | 14.31 | 13.78 | 1.846 | 0.058 | 34.6 | 165.4 | 200 |
| 19 | 7F | NS02001647 | 1 | 0.44 | 0.43 | 0.55 | 0.53 | 0.63 | 0.61 | 1.032 | 0.027 | 74.1 | 125.9 | 200 |
| 20 | 7G | NS02001832 | 1 | 0.27 | 0.19 | 0.65 | 0.51 | 0.79 | 0.65 | 1.300 | 0.028 | 70.3 | 129.7 | 200 |
| 21 | 8B | NS02001967 | 1 | 22.05 | 20.95 | 22.70 | 21.51 | 22.76 | 21.58 | 1.867 | 0.125 | 16.1 | 183.9 | 200 |
| 22 | 8C | NS02001970 | 1 | 0.55 | 0.46 | 0.28 | 0.18 | 0.43 | 0.34 | 1.326 | 0.021 | 95.5 | 104.5 | 200 |
| 23 | 8H | NS02002359 | 1 | 19.13 | 18.36 | 20.03 | 19.26 | 20.18 | 19.39 | 2.121 | 0.074 | 27.2 | 172.8 | 200 |
| 24 | 9B | NS02002905 | 1 | 30.19 | 29.78 | 32.59 | 32.31 | 33.35 | 32.91 | 2.223 | 0.037 | 53.6 | 146.4 | 200 |
| 25 | 9C | NS02002906 | 1 | 17.36 | 16.68 | 17.67 | 16.99 | 17.79 | 17.09 | 1.778 | 0.079 | 25.5 | 174.5 | 200 |
| 26 | 9E | NS02003306 | 1A | 12.49 | 12.19 | 12.57 | 12.30 | 12.49 | 12.22 | 2.061 | 0.027 | 73.6 | 126.4 | 200 |
| 27 | 9F | NS02003307 | 1B | 11.05 | 10.80 | 11.15 | 10.91 | 11.18 | 10.94 | 2.423 | 0.021 | 97.6 | 102.4 | 200 |
| 28 | 9G | NS02003409 | 1 | 17.09 | 16.41 | 17.33 | 16.66 | 17.40 | 16.72 | 1.875 | 0.073 | 27.6 | 172.4 | 200 |
| 29 | 10A | NS02003610 | 1 | 15.19 | 14.56 | 15.42 | 14.80 | 15.49 | 14.80 | 1.925 | 0.067 | 29.7 | 170.3 | 200 |
| 30 | 10B | NS02003612 | 1 | 17.95 | 17.52 | 18.34 | 17.86 | 18.37 | 17.91 | 1.643 | 0.058 | 34.3 | 165.7 | 200 |
| 31 | 10C | NS02003710 | 1 | 21.64 | 20.68 | 21.55 | 20.64 | 21.44 | 20.56 | 2.015 | 0.091 | 22.0 | 178.0 | 200 |
| 32 | 10D | NS02003712 | 1 | 1.00 | 1.11 | 0.78 | 0.93 | 0.69 | 0.85 | 0.851 | 0.041 | 48.6 | 151.4 | 200 |
| 33 | 10E | NS02003914 | 1 | 25.91 | 24.90 | 26.27 | 25.30 | 26.10 | 25.11 | 1.614 | 0.129 | 15.5 | 184.5 | 200 |
| 34 | 10G | NS02004435 | 1 | 10.94 | 10.69 | 11.03 | 10.78 | 11.00 | 10.77 | 2.406 | 0.021 | 96.0 | 104.0 | 200 |
| 35 | 11A | NS02004521 | 1 | 16.17 | 15.74 | 16.31 | 15.87 | 16.34 | 15.89 | 1.733 | 0.052 | 38.5 | 161.5 | 200 |
| 36 | 11E | NS02005343 | 1 | 18.19 | 17.57 | 18.40 | 17.75 | 19.41 | 17.76 | 1.615 | 0.084 | 23.8 | 176.2 | 200 |
| 37 | 11G | NS02005565 | 1 | 2.23 | 2.03 | 2.34 | 2.13 | 2.69 | 2.49 | 1.094 | 0.121 | 16.5 | 183.5 | 200 |
| 38 | 11H | NS02005568 | 1 | 3.96 | 3.93 | 0.96 | 0.92 | 1.21 | 1.13 | 1.041 | 0.102 | 19.6 | 180.4 | 200 |
| 39 | 12B | NS02006000 | 1A | 2.20 | 2.12 | 3.77 | 3.67 | 4.38 | 4.25 | 1.032 | 0.173 | 11.6 | 188.4 | 200 |
| 40 | 12C | NS02006000 | 1B | | | 0.41 | 0.39 | 0.49 | 0.49 | 1.026 | 0.023 | 88.9 | 111.1 | 200 |
| 41 | 12E | NS02006074 | 1 | 3.32 | 3.54 | 3.59 | 3.84 | 3.71 | 3.97 | 0.936 | 0.177 | 11.3 | 188.7 | 200 |
| 42 | 13E | NS02006829 | 1 | 1.36 | 1.59 | 1.38 | 1.65 | 1.37 | 1.62 | 0.846 | 0.069 | 29.2 | 170.8 | 200 |
| 43 | 13F | NS02006965 | 1 | 1.45 | 1.72 | 1.44 | 1.70 | 1.41 | 1.69 | 0.841 | 0.072 | 27.9 | 172.1 | 200 |
| 44 | 14B | NS02007115 | 1 | 7.05 | 7.01 | 7.30 | 7.21 | 7.28 | 7.19 | 1.010 | 0.361 | 5.5 | 194.5 | 200 |
| 45 | 14D | NS02007455 | 1B | 0.70 | 0.72 | 0.66 | 0.70 | 0.64 | 0.69 | 0.948 | 0.033 | 60.0 | 140.0 | 200 |
| 46 | 14E | NS02007591 | 1A | 4.5 | 4.25 | 3.93 | 3.61 | 2.63 | 2.39 | 0.941 | 0.171 | 11.7 | 188.3 | 200 |
| 47 | 14F | NS02007598 | 1 | 4.31 | 4.24 | 4.60 | 4.54 | 4.64 | 4.61 | 1.012 | 0.226 | 8.9 | 191.1 | 200 |
| 48 | 14G | NS02007665 | 1 | 21.75 | 21.34 | 22.59 | 22.16 | 22.67 | 22.27 | 1.019 | 1.117 | 1.8 | 198.2 | 200 |
| 49 | 15B | NS02007899 | 1 | 1.31 | 1.32 | 1.37 | 1.41 | 1.44 | 1.48 | 0.979 | 0.069 | 29.1 | 170.9 | 200 |
| 50 | 15D | NS02008655 | 1A | 11.26 | 11.48 | 13.59 | 13.77 | 14.35 | 14.54 | 0.985 | 0.653 | 3.1 | 196.9 | 200 |
| 51 | 15F | NS02008759 | 1 | 9.33 | 9.43 | 10.85 | 10.92 | 11.28 | 11.42 | 0.990 | 0.524 | 3.8 | 196.2 | 200 |
| 52 | 15H | NS02009377 | 1 | 8.76 | 8.86 | 10.38 | 10.57 | 10.92 | 11.09 | 0.985 | 0.501 | 4.0 | 196.0 | 200 |
| 53 | 16A | NS02009378 | 1 | 1.58 | 1.58 | 1.75 | 1.77 | 1.80 | 1.84 | 0.989 | 0.086 | 23.4 | 176.6 | 200 |

# Appendix 31 (Continued)

DNA purity and DNA yield of 84 extracted DNA samples (Benign)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA purity | DNA yield (μg/μl) | Dilution for PCR (10 ng/μl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | | | DNA | $dH_2O$ | Total |
| 54 | 16B | NS02009385 | 1 | 0.23 | 0.23 | 0.27 | 0.29 | 0.28 | 0.30 | 0.933 | 0.013 | 155.4 | 44.6 | 200 |
| 55 | 16C | NS02009890 | 1 | 0.60 | 0.60 | 0.56 | 0.59 | 0.51 | 0.54 | 0.964 | 0.028 | 71.9 | 128.1 | 200 |
| 56 | 16D | NS02009892 | 1 | 0.53 | 0.49 | 0.47 | 0.45 | 0.42 | 0.42 | 1.042 | 0.024 | 84.5 | 115.5 | 200 |
| 57 | 16E | NS02010172 | 1 | 0.69 | 0.67 | 0.70 | 0.71 | 0.68 | 0.71 | 0.991 | 0.035 | 58.0 | 142.0 | 200 |
| 58 | 16F | NS02010172 | 2 | 4.40 | 4.36 | 4.56 | 4.54 | 4.42 | 4.41 | 1.005 | 0.223 | 9.0 | 191.0 | 200 |
| 59 | 16G | NS02010245 | 1 | 0.70 | 0.68 | 0.65 | 0.64 | 0.61 | 0.62 | 1.010 | 0.033 | 61.2 | 138.8 | 200 |
| 60 | 16H | NS02010301 | 1 | 1.10 | 1.09 | 1.00 | 1.00 | 0.95 | 0.96 | 1.000 | 0.051 | 39.3 | 160.7 | 200 |
| 61 | 17B | NS02010409 | 1 | 0.99 | 0.94 | 0.99 | 0.95 | 0.95 | 0.92 | 1.043 | 0.049 | 41.0 | 159.0 | 200 |
| 62 | 17C | NS02010583 | 1 | 0.49 | 0.48 | 0.44 | 0.43 | 0.41 | 0.41 | 1.015 | 0.022 | 89.6 | 110.4 | 200 |
| 63 | 17E | NS02010835 | 1 | 26.42 | 25.97 | 26.43 | 25.96 | 26.11 | 25.71 | 1.017 | 1.313 | 1.5 | 198.5 | 200 |
| 64 | 17F | NS02010904 | 1 | 0.76 | 0.73 | 0.71 | 0.71 | 0.72 | 0.72 | 1.014 | 0.037 | 54.8 | 145.2 | 200 |
| 65 | 18E | NS02011779 | 1 | 0.69 | 0.52 | 0.65 | 0.50 | 0.64 | 0.50 | 1.302 | 0.033 | 60.6 | 139.4 | 200 |
| 66 | 18F | NS02012088 | 1A | 0.47 | 0.34 | 0.42 | 0.31 | 0.44 | 0.33 | 1.363 | 0.022 | 90.2 | 109.8 | 200 |
| 67 | 19A | NS02012381 | 1 | 0.72 | 0.69 | 0.73 | 0.69 | 0.73 | 0.71 | 1.043 | 0.036 | 55.0 | 145.0 | 200 |
| 68 | 19B | NS02012538 | 1A | 19.77 | 19.34 | 19.61 | 19.21 | 19.62 | 19.23 | 1.021 | 0.983 | 2.0 | 198.0 | 200 |
| 69 | 19C | NS02012753 | 1 | 0.37 | 0.32 | 0.36 | 0.32 | 0.36 | 0.32 | 1.149 | 0.018 | 110.1 | 89.9 | 200 |
| 70 | 19F | NS02013040 | 1 | 0.45 | 0.45 | 0.47 | 0.48 | 0.50 | 0.53 | 0.974 | 0.024 | 84.5 | 115.5 | 200 |
| 71 | 20A | NS02013634 | 1B | 0.29 | 0.18 | 0.42 | 0.28 | 0.55 | 0.38 | 1.533 | 0.021 | 95.4 | 104.6 | 200 |
| 72 | 20B | NS02013654 | 1 | 2.25 | 2.15 | 2.24 | 2.14 | 1.69 | 1.56 | 0.945 | 0.098 | 20.5 | 179.5 | 200 |
| 73 | 20D | NS02013773 | 1 | 21.88 | 21.36 | 23.09 | 22.68 | 22.96 | 22.62 | 1.019 | 1.132 | 1.8 | 198.2 | 200 |
| 74 | 20E | NS02013919 | 1 | 1.46 | 0.95 | 1.73 | 1.22 | 2.05 | 1.51 | 1.438 | 0.087 | 22.9 | 177.1 | 200 |
| 75 | 21A | NS02014375 | 1 | 20.58 | 19.90 | 19.87 | 19.25 | 19.66 | 19.06 | 1.032 | 1.002 | 2.0 | 198.0 | 200 |
| 76 | 21C | NS02014462 | 1 | 1.44 | 1.43 | 1.53 | 1.44 | 1.23 | 1.13 | 0.951 | 0.067 | 30.0 | 170.0 | 200 |
| 77 | 21E | NS02014538 | 1 | 21.11 | 20.67 | 20.63 | 20.14 | 20.31 | 19.89 | 1.022 | 1.034 | 1.9 | 198.1 | 200 |
| 78 | 21F | NS02014854 | 1 | 2.52 | 2.74 | 1.89 | 2.12 | 1.19 | 1.29 | 0.911 | 0.093 | 21.4 | 178.6 | 200 |
| 79 | 21H | NS02015136 | 1 | 0.35 | 0.23 | 0.60 | 0.46 | 0.79 | 0.65 | 1.338 | 0.029 | 69.0 | 131.0 | 200 |
| 80 | 22F | NS02017209 | 1 | 1.19 | 1.15 | 0.94 | 0.99 | 1.25 | 1.31 | 1.022 | 0.058 | 34.8 | 165.2 | 200 |
| 81 | 22G | NS02017309 | 1A | 28.48 | 28.20 | 31.82 | 31.48 | 31.45 | 31.21 | 1.010 | 1.529 | 1.3 | 198.7 | 200 |
| 82 | 23A | NS02017375 | 2 | 1.21 | 1.28 | 2.08 | 2.09 | 3.11 | 3.06 | 0.990 | 0.107 | 18.8 | 181.3 | 200 |
| 83 | 23G | NS02018054 | 1 | 24.51 | 24.11 | 25.66 | 25.31 | 23.31 | 22.98 | 1.015 | 1.225 | 1.6 | 198.4 | 200 |
| 84 | 23H | NS02018368 | 4F | 25.23 | 24.68 | 25.76 | 25.28 | 23.96 | 23.49 | 1.020 | 1.249 | 1.6 | 198.4 | 200 |

# Appendix 32

## DNA purity and DNA yield of 12 extracted DNA samples (Dysplasia)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA | DNA yield | Dilution for PCR (10 ng/µl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | purity | (µg/µl) | DNA | $dH_2O$ | Total |
| 1 | 8A | NS02001962 | 1D | 4.49 | 4.58 | 4.55 | 4.60 | 4.53 | 4.64 | 0.982 | 0.226 | 8.8 | 191.2 | 200 |
| 2 | 9A | NS02002447 | 1 | 20.25 | 19.83 | 20.66 | 20.21 | 20.68 | 20.23 | 1.617 | 0.058 | 34.7 | 165.3 | 200 |
| 3 | 13H | NS02007111 | 1A | 1.12 | 1.27 | 1.47 | 1.64 | 1.48 | 1.69 | 0.885 | 0.068 | 29.5 | 170.5 | 200 |
| 4 | 14H | NS02007740 | 1 | 0.40 | 0.39 | 0.49 | 0.48 | 0.52 | 0.51 | 1.022 | 0.024 | 85.1 | 114.9 | 200 |
| 5 | 18C | NS02011734 | 1 | 1.40 | 1.07 | 1.39 | 1.07 | 1.35 | 1.05 | 1.298 | 0.069 | 29.0 | 171.0 | 200 |
| 6 | 18H | NS02012233 | 1 | 24.20 | 23.86 | 24.48 | 24.15 | 24.44 | 24.12 | 1.014 | 1.219 | 1.6 | 198.4 | 200 |
| 7 | 19H | NS02013597 | 1 | 0.35 | 0.31 | 0.57 | 0.51 | 0.63 | 0.55 | 1.132 | 0.026 | 77.4 | 122.6 | 200 |
| 8 | 21G | NS02014921 | 1 | 2.77 | 2.58 | 1.48 | 1.3 | 1.18 | 1.02 | 0.891 | 0.082 | 24.5 | 175.5 | 200 |
| 9 | 22B | NS02015903 | 1 | 1.45 | 1.32 | 1.36 | 1.19 | 0.95 | 0.81 | 0.879 | 0.055 | 36.1 | 163.9 | 200 |
| 10 | 22E | NS02016901 | 8D | 30.12 | 29.71 | 28.21 | 27.77 | 26.24 | 25.82 | 1.015 | 1.409 | 1.4 | 198.6 | 200 |
| 11 | 22H | NS02017374 | 1 | 1.38 | 1.38 | 1.60 | 1.57 | 2.05 | 2.00 | 1.015 | 0.084 | 23.9 | 176.1 | 200 |
| 12 | 23B | NS02017558 | 1 | 26.80 | 26.29 | 27.58 | 27.05 | 26.60 | 26.14 | 1.019 | 1.350 | 1.5 | 198.5 | 200 |

# Appendix 33

DNA purity and DNA yield of 80 extracted DNA samples (Carcinoma)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA | DNA yield | Dilution for PCR (10 ng/µl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | purity | (µg/µl) | DNA | $dH_2O$ | Total |
| 1 | 1B | NS01017460 | 5J | 6.84 | 6.61 | 6.75 | 6.53 | 6.62 | 6.40 | 1.712 | 0.027 | 75.0 | 125.0 | 200 |
| 2 | 1F | NS01017826 | 4D | 17.97 | 17.06 | 17.93 | 17.07 | 17.81 | 16.95 | 2.439 | 0.073 | 27.3 | 172.7 | 200 |
| 3 | 2F | NS02000316 | 7C | 21.12 | 20.20 | 21.51 | 20.65 | 21.52 | 20.69 | 2.075 | 0.084 | 23.8 | 176.2 | 200 |
| 4 | 3B | NS02001012 | 1A | 10.52 | 10.29 | 10.60 | 10.35 | 10.62 | 10.36 | 1.954 | 0.025 | 79.5 | 120.5 | 200 |
| 5 | 3C | NS02001012 | 1B | 14.22 | 13.59 | 13.14 | 12.66 | 12.81 | 12.34 | 2.082 | 0.051 | 39.3 | 160.7 | 200 |
| 6 | 3D | NS02001012 | 1C | 1.16 | 0.69 | 1.28 | 0.91 | 1.68 | 1.28 | 0.689 | 0.048 | 41.7 | 158.3 | 200 |
| 7 | 3E | NS02001012 | 1D | 11.30 | 10.94 | 11.34 | 11.00 | 11.34 | 11.00 | 1.912 | 0.036 | 55.0 | 145.0 | 200 |
| 8 | 3F | NS02001012 | 1E | 4.31 | 4.17 | 4.32 | 4.18 | 4.31 | 4.18 | 2.391 | 0.012 | 168.5 | 31.5 | 200 |
| 9 | 3G | NS02001012 | 1F | 12.75 | 12.35 | 12.88 | 12.47 | 12.91 | 12.51 | 1.720 | 0.048 | 41.5 | 158.5 | 200 |
| 10 | 3H | NS02001012 | 1G | 28.72 | 28.49 | 30.64 | 30.46 | 30.90 | 30.57 | 1.717 | 0.030 | 66.7 | 133.3 | 200 |
| 11 | 4A | NS02001012 | 1H | 8.09 | 7.95 | 8.12 | 7.98 | 8.11 | 7.97 | 1.902 | 0.015 | 134.2 | 65.8 | 200 |
| 12 | 4B | NS02001012 | 1I | 8.57 | 8.39 | 8.59 | 8.42 | 8.57 | 8.40 | 1.810 | 0.019 | 103.4 | 96.6 | 200 |
| 13 | 4C | NS02001012 | 1J | 0.95 | 1.05 | 1.32 | 1.43 | 1.6 | 1.71 | 1.086 | 0.070 | 28.6 | 171.4 | 200 |
| 14 | 4D | NS02001012 | 1K | 16.56 | 15.96 | 16.88 | 16.25 | 16.92 | 16.30 | 1.723 | 0.074 | 27.2 | 172.8 | 200 |
| 15 | 4E | NS02001012 | 1L | 13.59 | 13.32 | 13.77 | 13.51 | 13.79 | 13.52 | 1.640 | 0.034 | 58.5 | 141.5 | 200 |
| 16 | 4F | NS02001012 | 1M | 0.56 | 0.68 | 0.72 | 0.84 | 0.93 | 1.03 | 1.163 | 0.043 | 47.1 | 152.9 | 200 |
| 17 | 4G | NS02001012 | 1N | 16.26 | 15.72 | 16.69 | 16.17 | 16.77 | 16.26 | 1.748 | 0.061 | 32.7 | 167.3 | 200 |
| 18 | 4H | NS02001012 | 1O | 11.14 | 10.93 | 11.25 | 11.05 | 11.33 | 11.10 | 2.044 | 0.021 | 96.8 | 103.2 | 200 |
| 19 | 5A | NS02001012 | 1P | 11.73 | 11.43 | 12.55 | 12.27 | 12.60 | 12.33 | 1.715 | 0.034 | 58.8 | 141.2 | 200 |
| 20 | 5B | NS02001012 | 2A | 6.30 | 6.28 | 6.26 | 6.27 | 6.23 | 6.22 | 1.001 | 0.313 | 6.4 | 193.6 | 200 |
| 21 | 5C | NS02001012 | 2B | 20.28 | 19.42 | 20.56 | 19.66 | 20.76 | 19.84 | 1.993 | 0.090 | 22.3 | 177.7 | 200 |
| 22 | 5D | NS02001012 | 2C | 12.64 | 12.16 | 12.79 | 12.29 | 12.84 | 12.35 | 1.782 | 0.056 | 35.8 | 164.2 | 200 |
| 23 | 5E | NS02001012 | 2D | 16.94 | 16.23 | 17.24 | 16.50 | 17.25 | 16.55 | 1.757 | 0.083 | 24.0 | 176.0 | 200 |
| 24 | 5F | NS02001012 | 2E | 8.60 | 8.40 | 8.64 | 8.45 | 8.66 | 8.46 | 1.825 | 0.022 | 92.3 | 107.7 | 200 |
| 25 | 5G | NS02001012 | 2F | 8.55 | 8.39 | 8.63 | 8.45 | 8.64 | 8.47 | 1.796 | 0.020 | 101.7 | 98.3 | 200 |
| 26 | 5H | NS02001012 | 2G | 14.90 | 14.34 | 15.10 | 14.53 | 15.12 | 14.56 | 1.853 | 0.061 | 32.7 | 167.3 | 200 |
| 27 | 6A | NS02001012 | 2H | 16.40 | 15.76 | 16.65 | 16.00 | 16.70 | 16.06 | 1.919 | 0.067 | 29.8 | 170.2 | 200 |
| 28 | 6B | NS02001012 | 2I | 15.18 | 14.60 | 15.58 | 14.99 | 15.66 | 15.09 | 1.973 | 0.059 | 34.0 | 166.0 | 200 |
| 29 | 6C | NS02001012 | 2J | 6.08 | 5.94 | 6.09 | 5.97 | 6.10 | 5.98 | 1.801 | 0.014 | 139.5 | 60.5 | 200 |
| 30 | 6D | NS02001012 | 2K | 17.62 | 16.90 | 17.85 | 17.14 | 17.94 | 17.22 | 1.689 | 0.088 | 22.8 | 177.2 | 200 |
| 31 | 6E | NS02001012 | 2L | 19.49 | 18.56 | 19.65 | 18.76 | 19.74 | 18.86 | 1.726 | 0.107 | 18.7 | 181.3 | 200 |
| 32 | 8D | NS02002097 | 1 | 16.22 | 15.48 | 16.39 | 15.61 | 16.40 | 15.66 | 1.753 | 0.088 | 22.8 | 177.2 | 200 |
| 33 | 8E | NS02002098 | 1 | 11.44 | 11.11 | 11.42 | 11.11 | 11.42 | 11.12 | 2.189 | 0.029 | 69.4 | 130.6 | 200 |
| 34 | 8F | NS02002098 | 2 | 1.06 | 0.63 | 1.02 | 0.60 | 1.04 | 0.63 | 1.678 | 0.052 | 38.5 | 161.5 | 200 |
| 35 | 8G | NS02002185 | 1 | 20.15 | 19.21 | 20.23 | 19.31 | 20.24 | 19.32 | 1.733 | 0.110 | 18.3 | 181.7 | 200 |
| 36 | 9D | NS02003275 | 3J | 24.43 | 23.03 | 25.02 | 23.61 | 25.13 | 23.73 | 2.277 | 0.125 | 16.0 | 184.0 | 200 |
| 37 | 9H | NS02003552 | 3A | 1.93 | 1.44 | 1.90 | 1.43 | 1.92 | 1.48 | 1.322 | 0.096 | 20.9 | 179.1 | 200 |
| 38 | 10F | NS02004213 | 1 | 13.15 | 12.78 | 13.33 | 12.96 | 13.33 | 12.99 | 1.819 | 0.040 | 50.0 | 150.0 | 200 |
| 39 | 10H | NS02004442 | 3B | 22.37 | 21.32 | 22.42 | 21.42 | 22.38 | 21.36 | 1.760 | 0.119 | 16.9 | 183.1 | 200 |
| 40 | 11C | NS02005157 | 1 | 14.61 | 14.21 | 14.64 | 14.22 | 14.63 | 14.21 | 1.850 | 0.045 | 44.4 | 155.6 | 200 |
| 41 | 11D | NS02005229 | 3B | 18.20 | 17.46 | 18.35 | 17.64 | 18.40 | 17.68 | 1.668 | 0.090 | 22.1 | 177.9 | 200 |
| 42 | 11F | NS02005525 | 1C | 0.01 | 0.12 | 8.09 | 7.53 | 6.91 | 6.31 | 0.737 | 0.250 | 8.0 | 192.0 | 200 |
| 43 | 12A | NS02005642 | 1B | 18.06 | 17.36 | 18.36 | 17.68 | 18.42 | 17.72 | 1.981 | 0.071 | 28.3 | 171.7 | 200 |
| 44 | 12F | NS02006089 | 1 | 0.49 | 0.65 | 0.62 | 0.77 | 0.57 | 0.74 | 0.776 | 0.028 | 71.4 | 128.6 | 200 |
| 45 | 13A | NS02006419 | 1A | 3.89 | 3.85 | 3.81 | 3.82 | 3.78 | 3.79 | 1.001 | 0.191 | 10.5 | 189.5 | 200 |
| 46 | 13C | NS02006721 | 1B | 1.06 | 1.22 | 1.10 | 1.29 | 1.10 | 1.29 | 0.858 | 0.054 | 36.8 | 163.2 | 200 |
| 47 | 13D | NS02006728 | 3 | 1.79 | 2.11 | 1.90 | 2.20 | 1.89 | 2.21 | 0.856 | 0.093 | 21.5 | 178.5 | 200 |
| 48 | 13G | NS02007068 | 3A | 4.61 | 4.51 | 4.85 | 4.82 | 4.84 | 4.84 | 1.009 | 0.238 | 8.4 | 191.6 | 200 |
| 49 | 14A | NS02007112 | 1A | 2.86 | 3.07 | 2.89 | 3.13 | 2.87 | 3.12 | 0.925 | 0.144 | 13.9 | 186.1 | 200 |
| 50 | 14C | NS02007236 | 1C | 21.17 | 20.69 | 21.81 | 21.38 | 21.67 | 21.24 | 1.021 | 1.077 | 1.9 | 198.1 | 200 |
| 51 | 15A | NS02007766 | 1 | 2.32 | 2.33 | 2.57 | 2.60 | 3.04 | 3.05 | 0.994 | 0.132 | 15.1 | 184.9 | 200 |
| 52 | 15C | NS02008161 | 1A | 21.83 | 21.09 | 22.42 | 21.74 | 22.44 | 21.78 | 1.032 | 1.112 | 1.8 | 198.2 | 200 |
| 53 | 15E | NS02008741 | 1 | 5.76 | 5.92 | 6.84 | 6.97 | - | - | 0.977 | 0.315 | 6.3 | 193.7 | 200 |
| 54 | 15G | NS02009289 | 1 | 20.66 | 20.21 | 20.98 | 20.51 | 20.97 | 20.54 | 1.022 | 1.043 | 1.9 | 198.1 | 200 |
| 55 | 17A | NS02010352 | 1 | 1.32 | 0.87 | 1.30 | 0.86 | 1.23 | 0.81 | 1.516 | 0.064 | 31.2 | 168.8 | 200 |
| 56 | 17D | NS02010642 | 1A | 0.84 | 0.80 | 0.83 | 0.79 | 0.87 | 0.83 | 1.050 | 0.042 | 47.2 | 152.8 | 200 |
| 57 | 17G | NS02011108 | 1A | 20.35 | 19.92 | 20.97 | 20.55 | 21.04 | 20.64 | 1.020 | 1.039 | 1.9 | 198.1 | 200 |
| 58 | 17H | NS02011344 | 1 | 1.34 | 1.09 | 1.29 | 1.06 | 1.25 | 1.02 | 1.224 | 0.065 | 30.9 | 169.1 | 200 |
| 59 | 18A | NS02011372 | 1A | 1.11 | 0.88 | 1.05 | 0.82 | 0.89 | 0.67 | 1.290 | 0.051 | 39.3 | 160.7 | 200 |
| 60 | 18B | NS02011466 | 2A | 17.09 | 16.57 | 16.94 | 16.41 | 16.81 | 16.33 | 1.031 | 0.847 | 2.4 | 197.6 | 200 |

## Appendix 33 (Continued)

DNA purity and DNA yield of 80 extracted DNA samples (Carcinoma)

| | | | | Reading 1 | | Reading 2 | | Reading 3 | | DNA purity | DNA yield (μg/μl) | Dilution for PCR (10 ng/μl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Plate | Pathology no. | Block | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | $A_{260}$ | $A_{280}$ | | | DNA | $dH_2O$ | Total |
| 61 | 18D | NS02011761 | 1B | 0.37 | 0.29 | 0.34 | 0.29 | 0.34 | 0.29 | 1.218 | 0.018 | 114.3 | 85.7 | 200 |
| 62 | 18G | NS02012156 | 1 | 0.58 | 0.55 | 0.56 | 0.54 | 0.55 | 0.53 | 1.043 | 0.028 | 71.0 | 129.0 | 200 |
| 63 | 19D | NS02012797 | 1 | 0.54 | 0.43 | 0.55 | 0.46 | 0.52 | 0.45 | 1.203 | 0.027 | 74.5 | 125.5 | 200 |
| 64 | 19E | NS02012798 | 2 | 0.93 | 0.84 | 0.88 | 0.82 | 0.88 | 0.80 | 1.093 | 0.045 | 44.6 | 155.4 | 200 |
| 65 | 19G | NS02013296 | 3C | 24.05 | 23.22 | 21.42 | 20.65 | 20.70 | 19.98 | 1.036 | 1.103 | 1.8 | 198.2 | 200 |
| 66 | 20C | NS02013666 | 3O | 2.57 | 1.07 | 2.71 | 1.22 | 3.14 | 1.65 | 2.177 | 0.140 | 14.3 | 185.7 | 200 |
| 67 | 20F | NS02013948 | 1C | 2.37 | 1.98 | 2.76 | 2.33 | 3.17 | 2.74 | 1.183 | 0.139 | 14.4 | 185.6 | 200 |
| 68 | 20G | NS02014046 | 1D | 22.84 | 21.90 | 22.54 | 21.64 | 22.38 | 21.46 | 1.043 | 1.129 | 1.8 | 198.2 | 200 |
| 69 | 20H | NS02014101 | 1A | 25.37 | 24.96 | 24.96 | 24.54 | 24.53 | 24.10 | 1.017 | 1.247 | 1.6 | 198.4 | 200 |
| 70 | 21B | NS02014416 | 2B | 26.60 | 26.00 | 25.79 | 25.23 | 25.07 | 24.50 | 1.023 | 1.291 | 1.5 | 198.5 | 200 |
| 71 | 21D | NS02014490 | 1A | 25.48 | 24.58 | 24.54 | 23.68 | 24.16 | 23.30 | 1.037 | 1.236 | 1.6 | 198.4 | 200 |
| 72 | 22A | NS02015366 | 1B | 23.54 | 23.19 | 23.08 | 22.78 | 22.76 | 22.50 | 1.013 | 1.156 | 1.7 | 198.3 | 200 |
| 73 | 22C | NS02016061 | 1 | 25.64 | 25.13 | 25.06 | 24.53 | 24.84 | 24.29 | 1.022 | 1.259 | 1.6 | 198.4 | 200 |
| 74 | 22D | NS02016362 | 2A | 29.10 | 28.30 | 28.63 | 27.79 | 27.60 | 26.81 | 1.029 | 1.422 | 1.4 | 198.6 | 200 |
| 75 | 23C | NS02017642 | 5Q | 25.73 | 25.07 | 26.89 | 26.35 | 26.73 | 26.22 | 1.021 | 1.322 | 1.5 | 198.5 | 200 |
| 76 | 23D | NS02017902 | 1 | 24.69 | 23.98 | 25.47 | 24.77 | 24.41 | 23.76 | 1.028 | 1.243 | 1.6 | 198.4 | 200 |
| 77 | 23E | NS02017946 | 2F | 25.82 | 25.28 | 26.34 | 25.78 | 26.10 | 25.63 | 1.020 | 1.304 | 1.5 | 198.5 | 200 |
| 78 | 23F | NS02018053 | 1 | 23.28 | 22.85 | 23.35 | 22.90 | 21.85 | 21.45 | 1.019 | 1.141 | 1.8 | 198.2 | 200 |
| 79 | 24A | NS02018494 | 1A | 25.92 | 25.52 | 28.56 | 28.09 | 25.88 | 25.45 | 1.017 | 1.339 | 1.5 | 198.5 | 200 |
| 80 | 24B | NS02018494 | 1B | 26.34 | 25.93 | 25.39 | 25.05 | 24.69 | 24.35 | 1.015 | 1.274 | 1.6 | 198.4 | 200 |

## Appendix 34

PCR results for β-globin and L1 of alpha HPV (Benign)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 1 | 1A | NS01006314 | 1 | + | - | + | + | + |
| 2 | 1C | NS01017677 | 2 | + | - | + | + | + |
| 3 | 1D | NS01017679 | 1 | + | - | + | + | + |
| 4 | 1E | NS01017750 | 1 | + | - | - | + | + |
| 5 | 1G | NS01018047 | 1 | + | - | + | + | + |
| 6 | 1H | NS01018047 | 2 | + | - | - | - | - |
| 7 | 2B | NS01018532 | 1 | + | - | + | + | + |
| 8 | 2C | NS01018560 | 1 | + | - | + | + | + |
| 9 | 2E | NS02000314 | 2 | + | - | + | + | + |
| 10 | 2G | NS02000370 | 1 | + | - | + | + | + |
| 11 | 2H | NS02000373 | 1 | + | - | + | + | + |
| 12 | 3A | NS02000961 | 1 | + | - | + | + | + |
| 13 | 6G | NS02001225 | 1 | + | - | - | - | - |
| 14 | 6H | NS01001286 | 1B | + | - | + | + | + |
| 15 | 7A | NS02001288 | 1 | + | - | + | + | + |
| 16 | 7B | NS02001290 | 1 | + | - | + | + | + |
| 17 | 7C | NS02001293 | 1 | + | - | - | - | - |
| 18 | 7D | NS02001294 | 1 | + | - | + | + | + |
| 19 | 7F | NS02001647 | 1 | + | - | - | + | + |
| 20 | 7G | NS02001832 | 1 | + | - | + | + | + |
| 21 | 8B | NS02001967 | 1 | + | - | + | + | + |
| 22 | 8C | NS02001970 | 1 | + | - | + | + | + |
| 23 | 8H | NS02002359 | 1 | + | - | + | + | + |
| 24 | 9B | NS02002905 | 1 | + | - | + | + | + |
| 25 | 9C | NS02002906 | 1 | + | - | + | + | + |
| 26 | 9E | NS02003306 | 1A | + | - | + | + | + |
| 27 | 9F | NS02003307 | 1B | + | - | + | + | + |
| 28 | 9G | NS02003409 | 1 | + | - | - | - | - |
| 29 | 10A | NS02003610 | 1 | + | - | + | + | + |
| 30 | 10B | NS02003612 | 1 | + | - | - | - | - |
| 31 | 10C | NS02003710 | 1 | + | - | + | + | + |
| 32 | 10D | NS02003712 | 1 | + | - | + | + | + |
| 33 | 10E | NS02003914 | 1 | + | - | + | + | + |
| 34 | 10G | NS02004435 | 1 | + | - | + | + | + |
| 35 | 11A | NS02004521 | 1 | + | - | + | + | + |
| 36 | 11E | NS02005343 | 1 | + | - | + | + | + |
| 37 | 11G | NS02005565 | 1 | + | - | + | + | + |
| 38 | 11H | NS02005568 | 1 | + | - | + | + | + |
| 39 | 12B | NS02006000 | 1A | + | - | + | + | + |
| 40 | 12C | NS02006000 | 1B | + | - | $+^{\varepsilon}$ | - | - |
| 41 | 12E | NS02006074 | 1 | + | - | + | + | + |
| | | | | | | +34 | +35 | +35 |

Note: + Positive PCR and - Negative PCR; $^{\varepsilon}$ - False positive PCR

## Appendix 34 (Continued)

PCR results for β-globin and L1 of alpha HPV (Benign)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 42 | 13E | NS02006829 | 1 | + | - | - | - | - |
| 43 | 13F | NS02006965 | 1 | + | - | - | - | - |
| 44 | 14B | NS02007115 | 1 | + | - | + | + | + |
| 45 | 14D | NS02007455 | 1B | + | - | + | + | + |
| 46 | 14E | NS02007591 | 1A | + | - | - | - | - |
| 47 | 14F | NS02007598 | 1 | + | - | - | - | - |
| 48 | 14G | NS02007665 | 1 | + | - | + | + | + |
| 49 | 15B | NS02007899 | 1 | + | - | + | + | + |
| 50 | 15D | NS02008655 | 1A | + | - | - | - | - |
| 51 | 15F | NS02008759 | 1 | + | - | + | + | + |
| 52 | 15H | NS02009377 | 1 | + | - | - | - | - |
| 53 | 16A | NS02009378 | 1 | + | - | + | + | + |
| 54 | 16B | NS02009385 | 1 | + | - | + | + | + |
| 55 | 16C | NS02009890 | 1 | + | - | + | + | + |
| 56 | 16D | NS02009892 | 1 | + | - | - | - | - |
| 57 | 16E | NS02010172 | 1 | + | - | $+^{\varepsilon}$ | - | - |
| 58 | 16F | NS02010172 | 2 | + | - | + | + | + |
| 59 | 16G | NS02010245 | 1 | + | - | - | - | - |
| 60 | 16H | NS02010301 | 1 | + | - | - | - | - |
| 61 | 17B | NS02010409 | 1 | + | - | $+^{\varepsilon}$ | - | - |
| 62 | 17C | NS02010583 | 1 | + | - | + | + | + |
| 63 | 17E | NS02010835 | 1 | + | - | + | + | + |
| 64 | 17F | NS02010904 | 1 | + | - | - | - | - |
| 65 | 18E | NS02011779 | 1 | + | - | $+^{\varepsilon}$ | - | - |
| 66 | 18F | NS02012088 | 1A | + | - | - | - | - |
| 67 | 19A | NS02012381 | 1 | + | - | - | - | - |
| 68 | 19B | NS02012538 | 1A | + | - | - | - | - |
| 69 | 19C | NS02012753 | 1 | + | - | - | - | - |
| 70 | 19F | NS02013040 | 1 | + | - | + | + | + |
| 71 | 20A | NS02013634 | 1B | + | - | + | + | + |
| 72 | 20B | NS02013654 | 1 | + | - | + | + | + |
| 73 | 20D | NS02013773 | 1 | + | - | + | + | + |
| 74 | 20E | NS02013919 | 1 | + | - | - | + | + |
| 75 | 21A | NS02014375 | 1 | + | - | + | + | + |
| 76 | 21C | NS02014462 | 1 | + | - | + | + | + |
| 77 | 21E | NS02014538 | 1 | + | - | + | + | + |
| 78 | 21F | NS02014854 | 1 | + | - | + | + | + |
| 79 | 21H | NS02015136 | 1 | + | - | - | + | + |
| 80 | 22F | NS02017209 | 1 | + | - | + | + | + |
| 81 | 22G | NS02017309 | 1A | + | - | + | + | + |
| 82 | 23A | NS02017375 | 2 | + | - | + | + | + |
| 83 | 23G | NS02018054 | 1 | + | - | + | + | + |
| 84 | 23H | NS02018368 | 4F | + | - | + | + | + |
| | | | | | | +27 | +26 | +26 |
| | | PCR positive (%): | | 84 (100.0) | 0 (0.0) | 61 (72.6) | 61 (72.6) | 61 (72.6) |
| | | PCR negative (%): | | 0 (0.0) | 84 (100.0) | 23 (27.4) | 23 (27.4) | 23 (27.4) |
| | | Total samples: | | 84 | 84 | 84 | 84 | 84 |

## Appendix 35

PCR amplification results for β-globin and L1 of alpha HPV (Dysplasia)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 1 | 8A | NS02001962 | 1D | + | - | + | + | + |
| 2 | 9A | NS02002447 | 1 | + | - | + | + | + |
| 3 | 13H | NS02007111 | 1A | + | - | + | + | + |
| 4 | 14H | NS02007740 | 1 | + | + | + | + | + |
| 5 | 18C | NS02011734 | 1 | + | - | - | - | - |
| 6 | 18H | NS02012233 | 1 | + | - | + | + | + |
| 7 | 19H | NS02013597 | 1 | + | - | + | + | + |
| 8 | 21G | NS02014921 | 1 | + | - | + | + | + |
| 9 | 22B | NS02015903 | 1 | + | - | + | + | + |
| 10 | 22E | NS02016901 | 8D | + | - | - | - | - |
| 11 | 22H | NS02017374 | 1 | + | - | + | + | + |
| 12 | 23B | NS02017558 | 1 | + | - | - | - | - |
| | | PCR positive (%): | | 12 (100.0) | 1 (8.3) | 9 (75.0) | 9 (75.0) | 9 (75.0) |
| | | PCR negative (%): | | 0 (0.0) | 11 (91.7) | 3 (25.0) | 3 (25.0) | 3 (25.0) |
| | | Total samples: | | 12 | 12 | 12 | 12 | 12 |

Note: + Positive PCR and - Negative PCR

## Appendix 36

PCR results for β-globin and L1 of alpha HPV (Carcinoma)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 1 | 1B | NS01017460 | 5J | + | - | - | - | - |
| 2 | 1F | NS01017826 | 4D | + | - | - | + | + |
| 3 | 2F | NS02000316 | 7C | + | - | + | + | + |
| 4 | 3B | NS02001012 | 1A | + | - | - | + | + |
| 5 | 3C | NS02001012 | 1B | + | - | - | + | + |
| 6 | 3D | NS02001012 | 1C | + | - | - | + | + |
| 7 | 3E | NS02001012 | 1D | + | - | - | + | + |
| 8 | 3F | NS02001012 | 1E | + | - | + | + | + |
| 9 | 3G | NS02001012 | 1F | + | - | + | + | + |
| 10 | 3H | NS02001012 | 1G | + | - | + | + | + |
| 11 | 4A | NS02001012 | 1H | + | - | + | + | + |
| 12 | 4B | NS02001012 | 1I | + | - | + | + | + |
| 13 | 4C | NS02001012 | 1J | + | - | + | + | + |
| 14 | 4D | NS02001012 | 1K | + | - | + | + | + |
| 15 | 4E | NS02001012 | 1L | + | - | + | + | + |
| 16 | 4F | NS02001012 | 1M | + | - | + | + | + |
| 17 | 4G | NS02001012 | 1N | + | - | + | + | + |
| 18 | 4H | NS02001012 | 1O | + | - | - | - | - |
| 19 | 5A | NS02001012 | 1P | + | - | + | + | + |
| 20 | 5B | NS02001012 | 2A | + | - | + | + | + |
| 21 | 5C | NS02001012 | 2B | + | - | + | + | + |
| 22 | 5D | NS02001012 | 2C | + | - | + | + | + |
| 23 | 5E | NS02001012 | 2D | + | - | + | + | + |
| 24 | 5F | NS02001012 | 2E | + | - | - | + | + |
| 25 | 5G | NS02001012 | 2F | + | - | + | + | + |
| 26 | 5H | NS02001012 | 2G | + | - | + | + | + |
| 27 | 6A | NS02001012 | 2H | + | - | + | + | + |
| 28 | 6B | NS02001012 | 2I | + | - | + | + | + |
| 29 | 6C | NS02001012 | 2J | + | - | + | + | + |
| 30 | 6D | NS02001012 | 2K | + | - | - | - | - |
| 31 | 6E | NS02001012 | 2L | + | - | - | - | - |
| 32 | 8D | NS02002097 | 1 | + | - | - | + | + |
| 33 | 8E | NS02002098 | 1 | + | - | + | + | + |
| 34 | 8F | NS02002098 | 2 | + | - | + | + | + |
| 35 | 8G | NS02002185 | 1 | + | - | + | + | + |
| 36 | 9D | NS02003275 | 3J | + | - | + | + | + |
| 37 | 9H | NS02003552 | 3A | + | - | + | + | + |
| 38 | 10F | NS02004213 | 1 | + | - | - | - | - |
| 39 | 10H | NS02004442 | 3B | + | - | + | + | + |
| 40 | 11C | NS02005157 | 1 | + | - | + | + | + |
| 41 | 11D | NS02005229 | 3B | + | - | + | + | + |
| 42 | 11F | NS02005525 | 1C | + | - | + | + | + |
| 43 | 12A | NS02005642 | 1B | + | - | + | + | + |
| 44 | 12F | NS02006089 | 1 | + | - | + | + | + |
| 45 | 13A | NS02006419 | 1A | + | - | - | + | + |
| 46 | 13C | NS02006721 | 1B | + | - | - | + | + |
| 47 | 13D | NS02006728 | 3 | + | - | + | + | + |

Note: + Positive PCR and - Negative PCR

## Appendix 36 (Continued)

PCR results for β-globin and L1 of alpha HPV (Carcinoma)

| No | ID | Pathology code | Block | β-globin (268 bp) | L1 α-HPV 450 bp | L1 α-HPV 190 bp | L1 α-HPV 140 bp | α-HPV Status |
|---|---|---|---|---|---|---|---|---|
| 48 | 13G | NS02007068 | 3A | + | - | + | + | + |
| 49 | 14A | NS02007112 | 1A | + | - | + | + | + |
| 50 | 14C | NS02007236 | 1C | + | - | + | + | + |
| 51 | 15A | NS02007766 | 1 | + | - | $+^{\varepsilon}$ | - | - |
| 52 | 15C | NS02008161 | 1A | + | - | + | + | + |
| 53 | 15E | NS02008741 | 1 | + | - | + | + | + |
| 54 | 15G | NS02009289 | 1 | + | - | + | + | + |
| 55 | 17A | NS02010352 | 1 | + | - | - | + | + |
| 56 | 17D | NS02010642 | 1A | + | - | + | + | + |
| 57 | 17G | NS02011108 | 1A | + | - | - | - | - |
| 58 | 17H | NS02011344 | 1 | + | - | + | + | + |
| 59 | 18A | NS02011372 | 1A | + | + | + | + | + |
| 60 | 18B | NS02011466 | 2A | + | - | - | - | - |
| 61 | 18D | NS02011761 | 1B | + | - | - | + | + |
| 62 | 18G | NS02012156 | 1 | + | - | + | + | + |
| 63 | 19D | NS02012797 | 1 | + | - | + | + | + |
| 64 | 19E | NS02012798 | 2 | + | - | + | + | + |
| 65 | 19G | NS02013296 | 3C | + | - | + | + | + |
| 66 | 20C | NS02013666 | 3O | + | - | - | + | + |
| 67 | 20F | NS02013948 | 1C | + | - | $+^{\varepsilon}$ | - | - |
| 68 | 20G | NS02014046 | 1D | + | - | - | + | + |
| 69 | 20H | NS02014101 | 1A | + | - | $+^{\varepsilon}$ | - | - |
| 70 | 21B | NS02014416 | 2B | + | - | + | + | + |
| 71 | 21D | NS02014490 | 1A | + | - | - | - | - |
| 72 | 22A | NS02015366 | 1B | + | - | + | + | + |
| 73 | 22C | NS02016061 | 1 | + | - | - | + | + |
| 74 | 22D | NS02016362 | 2A | + | - | + | + | + |
| 75 | 23C | NS02017642 | 5Q | + | - | - | - | - |
| 76 | 23D | NS02017902 | 1 | + | - | + | + | + |
| 77 | 23E | NS02017946 | 2F | + | - | + | + | + |
| 78 | 23F | NS02018053 | 1 | + | - | + | + | + |
| 79 | 24A | NS02018494 | 1A | + | - | - | - | - |
| 80 | 24B | NS02018494 | 1B | + | - | + | + | + |
| | | PCR positive (%): | | 80 (100.0) | 1 (1.3) | 56 (70.0) | 67 (83.8) | 67 (83.8) |
| | | PCR negative (%): | | 0 (0.0) | 79 (98.7) | 24 (30.0) | 13 (16.2) | 13 (16.2) |
| | | Total samples: | | 80 | 80 | 80 | 80 | 80 |

Note: + Positive PCR and - Negative PCR; $^{\varepsilon}$ - False positive PCR

# Appendix 37

## DNA sequencing and multiple sequences alignment results (Benign)

| No. | Plate | Pathology code | Block | Expected amplicon size (bp) | PCR purification methods[α] | HPV identification |
|---|---|---|---|---|---|---|
| 1 | 1C | NS01017677 | 2 | 140 | Gel | HPV 16 |
| 2 | 1G | NS01018047 | 1 | 140 | Gel | HPV 6 |
| 3 | 2B | NS01018532 | 1 | 140 | Gel | HPV 16 |
| 4 | 2C | NS01018560 | 1 | 190 | PCR | HPV 16 |
| 5 | 2E | NS02000314 | 2 | 190 | PCR | HPV 16 |
| 6 | 2G | NS02000370 | 1 | 140 | Gel | HPV 16 |
| 7 | 3A | NS02000961 | 1 | 190 | Gel | HPV 113 |
| 8 | 7A | NS02001288 | 1 | 190 | PCR | HPV 6 |
| 9 | 7B | NS02001290 | 1 | 140 | Gel | HPV 6 |
| 10 | 7F | NS02001647 | 1 | 140 | PCR | HPV 6 |
| 11 | 7G | NS02001832 | 1 | 140 | Gel | HPV 16 |
| 12 | 8B | NS02001967 | 1 | 140 | Gel | HPV 6 |
| 13 | 8C | NS02001970 | 1 | 140 | Gel | HPV 6 |
| 14 | 8H | NS02002359 | 1 | 140 | Gel | HPV 6 |
| 15 | 9B | NS02002905 | 1 | 140 | Gel | HPV 6 |
| 16 | 9C | NS02002906 | 1 | 140 | Gel | HPV 6 |
| 17 | 9E | NS02003306 | 1A | 140 | Gel | HPV 6 |
| 18 | 9F | NS02003307 | 1B | 140 | Gel | HPV 6 |
| 19 | 10D | NS02003712 | 1 | 190 | Gel | HPV 6 |
| 20 | 10E | NS02003914 | 1 | 190 | PCR | HPV 16 |
| 21 | 10G | NS02004435 | 1 | 140 | Gel | HPV 6 |
| 22 | 11E | NS02005343 | 1 | 190 | PCR | HPV 6 |
| 23 | 12B | NS02006000 | 1A | 140 | Gel | HPV 6 |
| 24 | 14B | NS02007115 | 1 | 190 | Gel | HPV 33 |
| 25 | 14D | NS02007455 | 1B | 190 | PCR | HPV 6 |
| 26 | 14G | NS02007665 | 1 | 190 | PCR | HPV 6 |
| 27 | 15F | NS02008759 | 1 | 190 | PCR | HPV 6 |
| 28 | 16F | NS02010172 | 2 | 140 | Gel | HPV 6 |
| 29 | 17C | NS02010583 | 1 | 140 | Gel | HPV 6 |
| 30 | 19F | NS02013040 | 1 | 140 | Gel | HPV 6 |
| 31 | 20A | NS02013634 | 1B | 140 | Gel | HPV 6 |
| 32 | 20B | NS02013654 | 1 | 190 | Gel | HPV 100 |
| 33 | 20D | NS02013773 | 1 | 140 | Gel | HPV 6 |
| 34 | 20E | NS02013919 | 1 | 140 | PCR | HPV 6 |
| 35 | 21A | NS02014375 | 1 | 140 | Gel | HPV 6 |
| 36 | 21C | NS02014462 | 1 | 140 | Gel | HPV 18 |
| 37 | 21E | NS02014538 | 1 | 140 | Gel | HPV 6 |
| 38 | 21H | NS02015136 | 1 | 140 | PCR | HPV 6 |
| 39 | 22F | NS02017209 | 1 | 140 | Gel | HPV 18 |
| 40 | 22G | NS02017309 | 1A | 190 | Gel | HPV 18 |
| 41 | 23G | NS02018054 | 1 | 140 | Gel | HPV 18 |
| 42 | 23H | NS02018368 | 4F | 140 | Gel | HPV 6 |
| 43 | 11H | NS02005568 | 1 | 140 | Gel | HPV 18 |
| 44 | 1A | NS01006314 | 1 | 140 | PCR | Negative |
| 45 | 1D | NS01017679 | 1 | 140 | PCR | Negative |
| 46 | 1E | NS01017750 | 1 | 190 | PCR | Negative |
| 47 | 2H | NS02000373 | 1 | 140 | PCR | Negative |
| 48 | 6H | NS01001286 | 1B | 190 | PCR | Negative |
| 49 | 7D | NS02001294 | 1 | 140 | Gel | Negative |
| 50 | 10A | NS02003610 | 1 | 140 | Gel | Negative |
| 51 | 10C | NS02003710 | 1 | 190 | Gel | Negative |
| 52 | 11A | NS02004521 | 1 | 140 | PCR | Negative |
| 53 | 11G | NS02005565 | 1 | 140 | PCR | Negative |
| 54 | 12E | NS02006074 | 1 | 140 | Gel | Negative |
| 55 | 15B | NS02007899 | 1 | 140 | PCR | Negative |
| 56 | 16A | NS02009378 | 1 | 140 | Gel | Negative |
| 57 | 16B | NS02009385 | 1 | 140 | Gel | Negative |
| 58 | 16C | NS02009890 | 1 | 140 | PCR | Negative |
| 59 | 17E | NS02010835 | 1 | 190 | PCR | Negative |
| 60 | 21F | NS02014854 | 1 | 190 | PCR | Negative |
| 61 | 23A | NS02017375 | 2 | 140 | Gel | Negative |

Notes:

[α] - PCR product purification methods: PCR - QIAquick PCR Purification Kit (Qiagen); Gel - QIAquick Gel Extraction Kit (Qiagen)

# Appendix 38

DNA sequencing and multiple sequences alignment results (Dysplasia)

| No. | Plate | Pathology code | Block | Expected amplicon size (bp) | PCR purification methods[α] | HPV identification |
|---|---|---|---|---|---|---|
| 1 | 14H | NS02007740 | 1 | 450 | Gel | HPV 100 |
| 2 | 18H | NS02012233 | 1 | 140 | Gel | HPV 6 |
| 3 | 19H | NS02013597 | 1 | 140 | Gel | HPV 6 |
| 4 | 21G | NS02014921 | 1 | 190 | PCR | HPV 16 |
| 5 | 22H | NS02017374 | 1 | 190 | Gel | HPV 100 |
| 6 | 8A | NS02001962 | 1D | 190 | Gel | Negative |
| 7 | 9A | NS02002447 | 1 | 140 | PCR | Negative |
| 8 | 13H | NS02007111 | 1A | 140 | PCR | Negative |
| 9 | 22B | NS02015903 | 1 | 190 | PCR | Negative |

Notes:

[α] - PCR product purification methods: PCR - QIAquick PCR Purification Kit (Qiagen); Gel - QIAquick Gel Extraction Kit (Qiagen)

# Appendix 39

## DNA sequencing and multiple sequences alignment results (Carcinoma)

| No. | Plate | Pathology code | Block | Expected amplicon size (bp) | PCR purification methods[a] | HPV identification |
|---|---|---|---|---|---|---|
| 1 | 1F | NS01017826 | 4D | 140 | Gel | HPV 6 |
| 2 | 2F | NS02000316 | 7C | 190 | PCR | HPV 16 |
| 3 | 3B | NS02001012 | 1A | 140 | Gel | HPV 6 |
| 4 | 3C | NS02001012 | 1B | 140 | Gel | HPV 16 |
| 5 | 3D | NS02001012 | 1C | 140 | PCR | HPV 6 |
| 6 | 3E | NS02001012 | 1D | 140 | Gel | HPV 6 |
| 7 | 3F | NS02001012 | 1E | 190 | PCR | HPV 16 |
| 8 | 3G | NS02001012 | 1F | 190 | PCR | HPV 16 |
| 9 | 3H | NS02001012 | 1G | 140 | Gel | HPV 16 |
| 10 | 4A | NS02001012 | 1H | 140 | Gel | HPV 16 |
| 11 | 4B | NS02001012 | 1I | 140 | Gel | HPV 16 |
| 12 | 4C | NS02001012 | 1J | 190 | PCR | HPV 16 |
| 13 | 4D | NS02001012 | 1K | 190 | PCR | HPV 16 |
| 14 | 4E | NS02001012 | 1L | 190 | PCR | HPV 16 |
| 15 | 5A | NS02001012 | 1P | 140 | Gel | HPV 109 |
| 16 | 5D | NS02001012 | 2C | 140 | Gel | HPV 103 |
| 17 | 5F | NS02001012 | 2E | 140 | Gel | HPV 6 |
| 18 | 5G | NS02001012 | 2F | 140 | Gel | HPV 6 |
| 19 | 8D | NS02002097 | 1 | 140 | Gel | HPV 6 |
| 20 | 8F | NS02002098 | 2 | 140 | Gel | HPV 6 |
| 21 | 8G | NS02002185 | 1 | 140 | Gel | HPV 6 |
| 22 | 11C | NS02005157 | 1 | 140 | Gel | HPV 6 |
| 23 | 11D | NS02005229 | 3B | 140 | Gel | HPV 18 |
| 24 | 11F | NS02005525 | 1C | 140 | Gel | HPV 6 |
| 25 | 13D | NS02006728 | 3 | 190 | Gel | HPV 64 |
| 26 | 14C | NS02007236 | 1C | 190 | PCR | HPV 6 |
| 27 | 15E | NS02008741 | 1 | 140 | Gel | HPV 6 |
| 28 | 15G | NS02009289 | 1 | 190 | PCR | HPV 6 |
| 29 | 17H | NS02011344 | 1 | 140 | Gel | HPV 6 |
| 30 | 18A | NS02011372 | 1A | 450 | Gel | HPV 100 |
| 31 | 18D | NS02011761 | 1B | 140 | PCR | HPV 6 |
| 32 | 19D | NS02012797 | 1 | 140 | Gel | HPV 6 |
| 33 | 19E | NS02012798 | 2 | 190 | Gel | HPV 100 |
| 34 | 19G | NS02013296 | 3C | 140 | Gel | HPV 6 |
| 35 | 20C | NS02013666 | 3O | 140 | Gel | HPV 18 |
| 36 | 20G | NS02014046 | 1D | 140 | PCR | HPV 18 |
| 37 | 21B | NS02014416 | 2B | 140 | Gel | HPV 6 |
| 38 | 22A | NS02015366 | 1B | 190 | PCR | HPV 6 |
| 39 | 23D | NS02017902 | 1 | 140 | Gel | HPV 6 |
| 40 | 23E | NS02017946 | 2F | 190 | PCR | HPV 6 |
| 41 | 23F | NS02018053 | 1 | 140 | Gel | HPV 6 |
| 42 | 24B | NS02018494 | 1B | 140 | Gel | HPV 6 |
| 43 | 13A | NS02006419 | 1A | 140 | Gel | Negative |
| 44 | 4F | NS02001012 | 1M | 140 | Gel | Negative |
| 45 | 4G | NS02001012 | 1N | 140 | PCR | Negative |
| 46 | 5B | NS02001012 | 2A | 140 | PCR | Negative |
| 47 | 5C | NS02001012 | 2B | 190 | PCR | Negative |
| 48 | 5E | NS02001012 | 2D | 140 | PCR | Negative |
| 49 | 5H | NS02001012 | 2G | 140 | Gel | Negative |
| 50 | 6A | NS02001012 | 2H | 140 | Gel | Negative |
| 51 | 6B | NS02001012 | 2I | 140 | Gel | Negative |
| 52 | 6C | NS02001012 | 2J | 140 | Gel | Negative |
| 53 | 8E | NS02002098 | 1 | 140 | Gel | Negative |
| 54 | 9D | NS02003275 | 3J | 140 | PCR | Negative |
| 55 | 9H | NS02003552 | 3A | 140 | PCR | Negative |
| 56 | 10H | NS02004442 | 3B | 140 | PCR | Negative |
| 57 | 12A | NS02005642 | 1B | 140 | PCR | Negative |
| 58 | 12F | NS02006089 | 1 | 140 | PCR | Negative |
| 59 | 13C | NS02006721 | 1B | 140 | Gel | Negative |
| 60 | 13G | NS02007068 | 3A | 190 | Gel | Negative |
| 61 | 14A | NS02007112 | 1A | 140 | PCR | Negative |
| 62 | 15C | NS02008161 | 1A | 140 | Gel | Negative |
| 63 | 17A | NS02010352 | 1 | 140 | Gel | Negative |
| 64 | 17D | NS02010642 | 1A | 140 | Gel | Negative |
| 65 | 18G | NS02012156 | 1 | 190 | Gel | Negative |
| 66 | 22C | NS02016061 | 1 | 140 | Gel | Negative |
| 67 | 22D | NS02016362 | 2A | 140 | Gel | Negative |

Notes:

[a] - PCR product purification methods: PCR - QIAquick PCR Purification Kit (Qiagen); Gel - QIAquick Gel Extraction Kit (Qiagen)

# Appendix 40

Table A: ANOVA and Independent-samples *t*-Test for means comparison

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) |
| Amplicon size | 0.028 | ANOVA | 0.241 | 0.045 | ANOVA | 0.263 |
| DNA Purification Kits | 0.000 | *t*-Test | 0.384 | 0.000 | *t*-Test | 0.000** |

*Notes:*
*$^{\alpha}$ - Levene's Test for Equality of Variances*
*$^{\beta}$ - Test for Equality of Means*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of 140bp > 450 bp ($p<0.01$) and190bp > 450 bp ($p<0.01$) and*
*QIAquick PCR Purification > QIAquick Gel Extraction ($p<0.001$)*

Table B: Kruskal-Wallis and Mann-Whitney Tests

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test$^{\alpha}$ | Asymp. sig. | Test$^{\alpha}$ | Asymp. sig. |
| Amplicon size | Kruskal-Wallis | 0.075 | Kruskal-Wallis | 0.020* |
| DNA Purification Kits | Mann-Whitney | 0.022* | Mann-Whitney | 0.000** |

*Notes:*
*$^{\alpha}$ - Test for Equality of Means*
*Cases less than two were excluded from Kruskal-Wallis Test*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of 140bp > 450 bp ($p<0.05$), 190bp > 450 bp ($p<0.05$) and QIAquick PCR kits > QIAquick Gel kits ($p<0.001$)*
*DNA purity of QIAquick PCR kits > QIAquick Gel kits ($p<0.05$)*

## Appendix 41

Table A: Means comparison of the purified amplicons and kit used in benign samples

| Parameters | $N$ | Mean ± $SD$ | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (ng/µl) |
| Amplicon size ($N$ = 61) | | | |
| 140 bp | 43 | 1.744 ± 0.312 | 30.16 ± 39.02 |
| 190 bp | 18 | 1.831 ± 0.150$^{\alpha}$ | 46.40 ± 40.86 |
| PCR Purification Kits ($N$ = 61) | | | |
| QIAquick PCR Purification | 22 | 1.808 ± 0.131$^{\alpha}$ | 78.48 ± 37.55$^{\alpha}$ |
| QIAquick Gel Extraction | 39 | 1.748 ± 0.331 | 10.39 ± 4.77 |

*Note: N, number of amplicons; SD, standard deviation; $^{\alpha}$ - Normal distribution data is assumed*

Table B: Independent-samples $t$-Test for means comparison (Benign)

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) |
| Amplicon size | 0.028 | $t$-Test | 0.262 | 0.830 | $t$-Test | 0.149 |
| DNA Purification Kits | 0.004 | $t$-Test | 0.417 | 0.000 | $t$-Test | 0.000** |

*Notes:*
*$^{\alpha}$ - Levene's Test for Equality of Variances*
*$^{\beta}$ - Test for Equality of Means*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of 140bp > 450 bp (p<0.01) and190bp > 450 bp (p<0.01) and*
*QIAquick PCR Purification > QIAquick Gel Extraction (p<0.001)*

Table C: Mann-Whitney Test for means comparison (Benign)

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test$^{\alpha}$ | Asymp. sig. | Test$^{\alpha}$ | Asymp. sig. |
| Amplicon size | Mann-Whitney | 0.074 | Mann-Whitney | 0.022* |
| DNA Purification Kits | Mann-Whitney | 0.084 | Mann-Whitney | 0.000** |

*Notes:*
*$^{\alpha}$ - Test for Equality of Means*
*Cases less than two were excluded from Kruskal-Wallis Test*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*190bp > 140 bp in DNA yield (p<0.05)*
*DNA yield of QIAquick PCR kits > QIAquick Gel kits (p<0.01)*

# Appendix 42

Table A: Means comparison of the purified amplicons and kit used in dysplasia samples

| Parameters | $N$ | Mean $\pm$ $SD$ | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (ng/µl) |
| Amplicon size ($N$ = 9) | | | |
| 140 bp | 4 | 1.819 $\pm$ 0.502 | 51.75 $\pm$ 48.99 |
| 190 bp | 4 | 1.792 $\pm$ 0.391$^{\alpha}$ | 35.34 $\pm$ 31.90 |
| 450 bp | 1 | 1.740 | 13.83 |
| PCR Purification Kits ($N$ = 9) | | | |
| QIAquick PCR Purification | 4 | 1.884 $\pm$ 0.531$^{\alpha}$ | 76.34 $\pm$ 26.96$^{\alpha}$ |
| QIAquick Gel Extraction | 5 | 1.625 $\pm$ 0.364 | 11.37 $\pm$ 2.04 |

*Note: N, number of amplicons; SD, standard deviation;* $^{\alpha}$ *Normal distribution data is assumed*

Table B: ANOVA and Independent-samples $t$-Test for means comparison (Dysplasia)

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) |
| Amplicon size | 0.688 | ANOVA | 0.505 | 0.094 | ANOVA | 0.695 |
| DNA Purification Kits | 0.757 | $t$-Test | 0.413 | 0.050 | $t$-Test | 0.001** |

*Notes:*

$^{\alpha}$ *- Levene's Test for Equality of Variances*

$^{\beta}$ *- Test for Equality of Means*

*** Statistically significant at the 0.01 level and * at the 0.05 level;*

*DNA yield of QIAquick PCR Purification > QIAquick Gel Extraction (p<0.001)*

Table C: Kruskal-Wallis and Mann-Whitney Tests (Dysplasia)

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test$^{\alpha}$ | Asymp. sig. | Test$^{\alpha}$ | Asymp. sig. |
| Amplicon size | Kruskal-Wallis | 0.291 | Kruskal-Wallis | 0.967 |
| DNA Purification Kits | Mann-Whitney | 0.556 | Mann-Whitney | 0.016** |

*Notes:*

$^{\alpha}$ *- Test for Equality of Means*

*Cases less than two were excluded from Kruskal-Wallis Test*

*** Statistically significant at the 0.01 level and * at the 0.05 level;*

*DNA yield of QIAquick PCR kits > QIAquick Gel kits (p<0.05)*

**Appendix 43**

Table A: Means comparison of the purified amplicons and kit used in carcinoma samples

| Parameters | $N$ | Mean $\pm$ $SD$ | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (ng/µl) |
| Amplicon size ($N$ = 67) | | | |
| 140 bp | 51 | $1.809 \pm 0.404$ | $35.29 \pm 40.60$ |
| 190 bp | 15 | $1.742 \pm 0.157^{\alpha}$ | $44.13 \pm 28.24$ |
| 450 bp | 1 | 1.575 | 8.30 |
| PCR Purification Kits ($N$ = 67) | | | |
| QIAquick PCR Purification | 23 | $1.800 \pm 0.130^{\alpha}$ | $79.06 \pm 34.31^{\alpha}$ |
| QIAquick Gel Extraction | 44 | $1.786 \pm 0.437$ | $14.81 \pm 12.82$ |

*Note: N, number of amplicons; SD, standard deviation; $^{\alpha}$ Normal distribution data is assumed*

Table B: ANOVA and Independent-samples $t$-Test for means comparison (Carcinoma)

| Parameter | DNA purity | | | DNA yield | | |
|---|---|---|---|---|---|---|
| | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) | Levene statistics$^{\alpha}$ (sig.) | Test$^{\beta}$ | Sig. (2-tailed) |
| Amplicon size | 0.037 | ANOVA | 0.689 | 0.101 | ANOVA | 0.556 |
| DNA Purification Kits | 0.001 | $t$-Test | 0.877 | 0.000 | $t$-Test | 0.000** |

*Notes:*
*$^{\alpha}$ - Levene's Test for Equality of Variances*
*$^{\beta}$ - Test for Equality of Means*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of QIAquick PCR Purification > QIAquick Gel Extraction ($p<0.001$)*

Table C: Kruskal-Wallis and Mann-Whitney Tests (Carcinoma)

| Parameter | DNA purity | | DNA yield | |
|---|---|---|---|---|
| | Test$^{\alpha}$ | Asymp. sig. | Test$^{\alpha}$ | Asymp. sig. |
| Amplicon size | Kruskal-Wallis | 0.646 | Kruskal-Wallis | 0.124 |
| DNA Purification Kits | Mann-Whitney | 0.168 | Mann-Whitney | 0.000** |

*Notes:*
*$^{\alpha}$ - Test for Equality of Means*
*Cases less than two were excluded from Kruskal-Wallis Test*
*** Statistically significant at the 0.01 level and * at the 0.05 level;*
*DNA yield of QIAquick PCR kits > QIAquick Gel kits ($p<0.001$)*

# Appendix 44

Table A: Crosstabs with Chi-square and Phi Analyses of HPV positivity among clinicopathological parameters

| Variable | Benign ($N$=84) | | | | | Dysplasia ($N$=12) | | | | | Carcinoma ($N$=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $N$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* | $N$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* | $n$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* |
| Gender | | | | 0.178 | 0.673 | | | | 1.656 | 0.198[α] | | | | 0.343 | 0.558 |
| Female | 45 | 24 | 21 | | | 5 | 1 | 4 | | | 49 | 27 | 22 | | |
| Male | 39 | 19 | 20 | | | 7 | 4 | 3 | | | 31 | 15 | 16 | | |
| Total | 84 | 43 | 41 | *df=1* | *Φ=(0.046)* | 12 | 5 | 7 | *df=1* | *Φ=0.371* | 80 | 42 | 38 | *df=1* | *Φ=(0.066)* |
| Age group | | | | 0.077 | 0.781 | | | | 3.360 | 0.067[α] | | | | 0.222 | 0.638 |
| 16-57y | 52 | 26 | 26 | | | 2 | 2 | 0 | | | 38 | 21 | 17 | | |
| 58-99y | 32 | 17 | 15 | | | 10 | 3 | 7 | | | 42 | 21 | 21 | | |
| Total | 84 | 43 | 41 | *df=1* | *Φ=0.030* | 12 | 5 | 7 | *df=1* | *Φ=(0.529)* | 80 | 42 | 38 | *df=1* | *Φ=(0.053)* |
| Disease outcome | | | | | | | | | | | | | | 0.029 | 0.866 |
| Alive | | | | | | | | | | | 35 | 18 | 17 | | |
| Dead | | | | | | | | | | | 45 | 24 | 21 | | |
| Total | | | | | | | | | | | 80 | 42 | 38 | *df=1* | *Φ=0.019* |
| Disease status | | | | N/A | N/A | | | | N/A | N/A | | | | 0.334 | 0.563 |
| No Recurrence | 84 | 43 | 41 | | | 12 | 5 | 7 | | | 75 | 40 | 35 | | |
| Recurrence | 0 | 0 | 0 | | | 0 | 0 | 0 | | | 5 | 2 | 3 | | |
| Total | 84 | 43 | 41 | | | 12 | 5 | 7 | | | 80 | 42 | 38 | *df=1* | *Φ=(0.065)* |
| DNA purity | | | | 0.178 | 0.673 | | | | 0.114 | 0.735[α] | | | | 1.074 | 0.300 |
| < 1.043 | 45 | 24 | 21 | | | 9 | 4 | 5 | | | 29 | 13 | 16 | | |
| 1.043 and above | 39 | 19 | 20 | | | 3 | 1 | 2 | | | 51 | 29 | 22 | | |
| Total | 84 | 43 | 41 | *df=1* | *Φ=(0.046)* | 12 | 5 | 7 | *df=1* | *Φ=(0.098)* | 80 | 42 | 38 | *df=1* | *Φ=0.116* |
| DNA yield | | | | 4.858* | 0.028 | | | | 0.343 | 0.558[α] | | | | 0.802 | 0.370 |
| < 0.074 μg/μl | 45 | 18 | 27 | | | 6 | 2 | 4 | | | 40 | 23 | 17 | | |
| 0.074 and above | 39 | 25 | 14 | | | 6 | 3 | 3 | | | 40 | 19 | 21 | | |
| Total | 84 | 43 | 41 | *df=1* | *Φ=(0.240)* | 12 | 5 | 7 | *df=1* | *Φ=0.169* | 80 | 42 | 38 | *df=1* | *Φ=(0.100)* |

Note: [α] generated from Fisher's Exact Test; *Φ* in parentheses to denote negative value

## Appendix 44 (continued)

Table B: Crosstabs with Chi-square and Cramer's V analyses of HPV positivity among clinicopathological parameters

| Variable | Benign ($N$=84) | | | | | Dysplasia ($N$=12) | | | | | Carcinoma ($N$=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $N$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* | $N$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* | $n$ | HPV detection positive | HPV detection negative | $\chi^2$ | *p value* |
| Anatomic site | | | | 5.946 | 0.745 | | | | 2.229 | 0.526 | | | | 6.754 | 0.344 |
| Tongue | 21 | 11 | 10 | | | - | - | - | | | 20 | 8 | 12 | | |
| Floor of the mouth | 3 | 1 | 2 | | | 8 | 3 | 5 | | | 12 | 8 | 4 | | |
| Soft palate | 3 | 2 | 1 | | | 2 | 1 | 1 | | | 34 | 19 | 15 | | |
| Retromolar pad | 1 | 1 | 0 | | | - | - | - | | | 3 | 0 | 3 | | |
| Lower lip | 4 | 2 | 2 | | | - | - | - | | | 4 | 2 | 2 | | |
| Buccal mucosa | 41 | 23 | 18 | | | 1 | 1 | 0 | | | 3 | 2 | 1 | | |
| Gingivae | 6 | 2 | 4 | | | - | - | - | | | 4 | 3 | 1 | | |
| Hard palate | 2 | 0 | 2 | | | 1 | 0 | 1 | | | - | - | - | | |
| Upper lip | 2 | 1 | 1 | | | - | - | - | | | - | - | - | | |
| Skin | 1 | 0 | 1 | | | - | - | - | | | - | - | - | | |
| Total | 84 | 43 | 41 | *df=9* | *CV=0.266*[α] | 12 | 5 | 7 | *df=3* | *CV=0.431*[α] | 80 | 42 | 38 | *df=6* | *CV=0.291*[α] |
| Tumour size | | | | | | | | | | | | | | 4.590 | 0.204 |
| T1 | | | | | | | | | | | 41 | 22 | 19 | | |
| T2 | | | | | | | | | | | 22 | 8 | 14 | | |
| T3 | | | | | | | | | | | 13 | 9 | 4 | | |
| T4 | | | | | | | | | | | 4 | 3 | 1 | | |
| Total | | | | | | | | | | | 80 | 42 | 38 | *df=3* | *CV=0.240*[α] |
| Regional lymph nodes | | | | | | | | | | | | | | 2.631 | 0.268 |
| N0 | | | | | | | | | | | 67 | 33 | 34 | | |
| N1 | | | | | | | | | | | 7 | 4 | 3 | | |
| N2 | | | | | | | | | | | 6 | 5 | 1 | | |
| Total | | | | | | | | | | | 80 | 42 | 38 | *df=2* | *CV=0.181*[α] |
| Differentiation | | | | | | | | | | | | | | 7.119 | 0.068 |
| Basal | | | | | | | | | | | 2 | 1 | 1 | | |
| Poor | | | | | | | | | | | 11 | 9 | 2 | | |
| Moderate | | | | | | | | | | | 58 | 30 | 28 | | |
| Well | | | | | | | | | | | 9 | 2 | 7 | | |
| Total | | | | | | | | | | | 80 | 42 | 38 | *df=3* | *CV=0.298*[α] |

Note: [α] generated from Cramer's V Test

# Appendix 45

Table A: Crosstabs with Chi-square and Phi Analyses of alpha HPV among clinicopathological parameters

| Variable | Benign ($N$=84) | | | | | Dysplasia ($N$=12) | | | | | Carcinoma ($N$=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | alpha HPV detection | | | | | alpha HPV detection | | | | | alpha HPV detection | | | |
| | *N* | Positive | Negative | $\chi^2$ | *p value* | *N* | positive | Negative | $\chi^2$ | *p value* | *n* | positive | negative | $\chi^2$ | *p value* |
| Gender | | | | 0.063 | 0.802 | | | | 2.857 | 0.091[α] | | | | 0.379 | 0.538 |
| Female | 45 | 22 | 23 | | | 5 | 0 | 5 | | | 49 | 24 | 25 | | |
| Male | 39 | 18 | 21 | | | 7 | 3 | 4 | | | 31 | 13 | 18 | | |
| Total | 84 | 40 | 44 | *df=1* | *Φ=(0.027)* | 12 | 3 | 9 | *df=1* | *Φ=0.488* | 80 | 37 | 43 | *df=1* | *Φ=(0.069)* |
| Age group | | | | 0.628 | 0.428 | | | | 0.800 | 0.371[α] | | | | 0.036 | 0.849 |
| 16-57y | 52 | 23 | 29 | | | 2 | 1 | 1 | | | 38 | 18 | 20 | | |
| 58-99y | 32 | 17 | 15 | | | 10 | 2 | 8 | | | 42 | 19 | 23 | | |
| Total | 84 | 40 | 44 | *df=1* | *Φ=0.086* | 12 | 3 | 9 | *df=1* | *Φ=(0.258)* | 80 | 37 | 43 | *df=1* | *Φ=(0.021)* |
| Disease outcome | | | | | | | | | | | | | | 0.007 | 0.932 |
| Alive | | | | | | | | | | | 35 | 16 | 19 | | |
| Dead | | | | | | | | | | | 45 | 21 | 24 | | |
| Total | | | | | | | | | | | 80 | 37 | 43 | *df=1* | *Φ=0.009* |
| Disease status | | | | N/A | N/A | | | | N/A | N/A | | | | 0.084 | 0.772[a] |
| No Recurrence | 84 | 40 | 44 | | | 12 | 3 | 9 | | | 75 | 35 | 40 | | |
| Recurrence | 0 | 0 | 0 | | | 0 | 0 | 0 | | | 5 | 2 | 3 | | |
| Total | 84 | 40 | 44 | | | 12 | 3 | 9 | | | 80 | 37 | 43 | *df=1* | *Φ=(0.032)* |
| DNA purity | | | | 0.063 | 0.802 | | | | 0.148 | 0.700[α] | | | | 0.434 | 0.510 |
| < 1.043 | 45 | 22 | 23 | | | 9 | 2 | 7 | | | 29 | 12 | 17 | | |
| 1.043 and above | 39 | 18 | 21 | | | 3 | 1 | 2 | | | 51 | 25 | 26 | | |
| Total | 84 | 40 | 44 | *df=1* | *Φ=(0.027)* | 12 | 3 | 9 | *df=1* | *Φ=(0.111)* | 80 | 37 | 43 | *df=1* | *Φ=0.074* |
| DNA yield | | | | 3.763 | 0.052 | | | | 0.444 | 0.505[α] | | | | 0.050 | 0.823 |
| < 0.074 μg/μl | 45 | 17 | 28 | | | 6 | 1 | 5 | | | 40 | 19 | 21 | | |
| 0.074 and above | 39 | 23 | 16 | | | 6 | 2 | 4 | | | 40 | 18 | 22 | | |
| Total | 84 | 40 | 44 | *df=1* | *Φ=(0.212)* | 12 | 3 | 9 | *df=1* | *Φ=0.192* | 80 | 37 | 43 | *df=1* | *Φ=(0.025)* |

Note: [α] generated from Fisher's Exact Test; *Φ* in parentheses to denote negative value

## Appendix 45 (continued)

Table B: Crosstabs with Chi-square and Cramer's V analyses of alpha HPV among clinicopathological parameters

| Variable | Benign (*N*=84) | | | | | Dysplasia (*N*=12) | | | | | Carcinoma (*N*=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | alpha HPV detection | | | | | alpha HPV detection | | | | | alpha HPV detection | | | |
| | *N* | positive | Negative | $\chi^2$ | *p value* | *N* | positive | Negative | $\chi^2$ | *p value* | *n* | positive | negative | $\chi^2$ | *p value* |
| Anatomic site | | | | 5.804 | 0.759 | | | | 1.333 | 0.721 | | | | 8.583 | 0.198 |
| Tongue | 21 | 9 | 12 | | | - | - | - | | | 20 | 6 | 14 | | |
| Floor of the mouth | 3 | 1 | 2 | | | 8 | 2 | 6 | | | 12 | 8 | 4 | | |
| Soft palate | 3 | 2 | 1 | | | 2 | 1 | 1 | | | 34 | 16 | 18 | | |
| Retromolar pad | 1 | 1 | 0 | | | - | - | - | | | 3 | 0 | 3 | | |
| Lower lip | 4 | 2 | 2 | | | - | - | - | | | 4 | 2 | 2 | | |
| Buccal mucosa | 41 | 22 | 19 | | | 1 | 0 | 1 | | | 3 | 2 | 1 | | |
| Gingivae | 6 | 2 | 4 | | | - | - | - | | | 4 | 3 | 1 | | |
| Hard palate | 2 | 0 | 2 | | | 1 | 0 | 1 | | | - | - | - | | |
| Upper lip | 2 | 1 | 1 | | | - | - | - | | | - | - | - | | |
| Skin | 1 | 0 | 1 | | | - | - | - | | | - | - | - | | |
| Total | 84 | 40 | 50 | *df=9* | *CV=0.263*$^{\alpha}$ | 12 | 5 | 7 | *df=3* | *CV=0.333*$^{\alpha}$ | 80 | 37 | 43 | *df=6* | *CV=0.328*$^{\alpha}$ |
| Tumour size | | | | | | | | | | | | | | 3.581 | 0.310 |
| T1 | | | | | | | | | | | 41 | 20 | 21 | | |
| T2 | | | | | | | | | | | 22 | 7 | 15 | | |
| T3 | | | | | | | | | | | 13 | 7 | 6 | | |
| T4 | | | | | | | | | | | 4 | 3 | 1 | | |
| Total | | | | | | | | | | | 80 | 37 | 43 | *df=3* | *CV=0.212*$^{\alpha}$ |
| Regional lymph nodes | | | | | | | | | | | | | | 4.189 | 0.123 |
| N0 | | | | | | | | | | | 67 | 28 | 39 | | |
| N1 | | | | | | | | | | | 7 | 4 | 3 | | |
| N2 | | | | | | | | | | | 6 | 5 | 1 | | |
| Total | | | | | | | | | | | 80 | 37 | 43 | *df=2* | *CV=0.229*$^{\alpha}$ |
| Differentiation | | | | | | | | | | | | | | 3.441 | 0.328 |
| Basal | | | | | | | | | | | 2 | 1 | 1 | | |
| Poor | | | | | | | | | | | 11 | 7 | 4 | | |
| Moderate | | | | | | | | | | | 58 | 27 | 31 | | |
| Well | | | | | | | | | | | 9 | 2 | 7 | | |
| Total | | | | | | | | | | | 80 | 37 | 43 | *df=3* | *CV=0.207*$^{\alpha}$ |

Note: $^{\alpha}$ generated from Cramer's V Test

## Appendix 46

Table A: Crosstabs with Chi-square and Phi Analyses of Low risk α-HPV among clinicopathological parameters

| Variable | Benign (*N*=84) | | | | | Dysplasia (*N*=12) | | | | | Carcinoma (*N*=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | LR α-HPV detection Positive | LR α-HPV detection Negative | $\chi^2$ | *p value* | *N* | LR α-HPV detection positive | LR α-HPV detection Negative | $\chi^2$ | *p value* | *n* | LR α-HPV detection positive | LR α-HPV detection negative | $\chi^2$ | *p value* |
| Gender | | | | 0.215 | 0.643 | | | | 1.714 | 0.190$^{\alpha}$ | | | | 0.725 | 0.395 |
| Female | 45 | 14 | 31 | | | 5 | 0 | 5 | | | 49 | 13 | 36 | | |
| Male | 39 | 14 | 25 | | | 7 | 2 | 5 | | | 31 | 11 | 20 | | |
| Total | 84 | 28 | 56 | *df=1* | *Φ=(0.051)* | 12 | 2 | 10 | *df=1* | *Φ=0.378* | 80 | 24 | 56 | *df=1* | *Φ=(0.095)* |
| Age group | | | | 0.101 | 0.751 | | | | 0.480 | 0.488$^{\alpha}$ | | | | 2.759 | 0.097 |
| 16-57y | 52 | 18 | 34 | | | 2 | 0 | 2 | | | 38 | 8 | 30 | | |
| 58-99y | 32 | 10 | 22 | | | 10 | 2 | 8 | | | 42 | 16 | 26 | | |
| Total | 84 | 28 | 56 | *df=1* | *Φ=(0.035)* | 12 | 2 | 10 | *df=1* | *Φ=(0.200)* | 80 | 24 | 56 | *df=1* | *Φ=(0.186)* |
| Disease outcome | | | | | | | | | | | | | | 2.963 | 0.085 |
| Alive | | | | | | | | | | | 35 | 14 | 21 | | |
| Dead | | | | | | | | | | | 45 | 10 | 35 | | |
| Total | | | | | | | | | | | 80 | 24 | 56 | *df=1* | *Φ=(0.192)* |
| Disease status | | | | N/A | N/A | | | | N/A | N/A | | | | 0.254 | 0.614$^{\alpha}$ |
| No Recurrence | 84 | 28 | 56 | | | 12 | 2 | 10 | | | 75 | 22 | 53 | | |
| Recurrence | 0 | 0 | 0 | | | 0 | 0 | 0 | | | 5 | 2 | 3 | | |
| Total | 84 | 28 | 56 | | | 12 | 2 | 10 | | | 80 | 24 | 56 | *df=1* | *Φ=(0.056)* |
| DNA purity | | | | 0.862 | 0.353 | | | | 0.800 | 0.371$^{\alpha}$ | | | | 2.805 | 0.094 |
| < 1.043 | 45 | 13 | 32 | | | 9 | 1 | 8 | | | 29 | 12 | 17 | | |
| 1.043 and above | 39 | 15 | 24 | | | 3 | 1 | 2 | | | 51 | 12 | 39 | | |
| Total | 84 | 28 | 56 | *df=1* | *Φ=(0.101)* | 12 | 2 | 10 | *df=1* | *Φ=(0.258)* | 80 | 24 | 56 | *df=1* | *Φ=(0.187)* |
| DNA yield | | | | 0.862 | 0.353 | | | | 0.000 | 1.000$^{\alpha}$ | | | | 0.238 | 0.626 |
| < 0.074 µg/µl | 45 | 13 | 32 | | | 6 | 1 | 5 | | | 40 | 11 | 29 | | |
| 0.074 and above | 39 | 15 | 24 | | | 6 | 1 | 5 | | | 40 | 13 | 27 | | |
| Total | 84 | 28 | 56 | *df=1* | *Φ=(0.101)* | 12 | 2 | 10 | *df=1* | *Φ=0.000* | 80 | 24 | 56 | *df=1* | *Φ=(0.055)* |

Note: $^{\alpha}$ generated from Fisher's Exact Test; *Φ* in parentheses to denote negative value

## Appendix 46 (continued)

Table B: Crosstabs with Chi-square and Cramer's V analyses of Low risk α-HPV among clinicopathological parameters

| Variable | Benign ($N$=84) | | | | | Dysplasia ($N$=12) | | | | | Carcinoma ($N$=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LR α-HPV | | | | | LR α-HPV | | | | | LR α-HPV | | | |
| | *N* | positive | Negative | $\chi^2$ | *p value* | *N* | positive | negative | $\chi^2$ | *p value* | *n* | Positive | negative | $\chi^2$ | *p value* |
| Anatomic site | | | | 8.195 | 0.515 | | | | 2.100 | 0.552 | | | | 8.688 | 0.192 |
| Tongue | 21 | 7 | 14 | | | - | - | - | | | 20 | 5 | 15 | | |
| Floor of the mouth | 3 | 1 | 2 | | | 8 | 1 | 7 | | | 12 | 6 | 6 | | |
| Soft palate | 3 | 2 | 1 | | | 2 | 1 | 1 | | | 34 | 7 | 27 | | |
| Retromolar pad | 1 | 1 | 0 | | | - | - | - | | | 3 | 0 | 3 | | |
| Lower lip | 4 | 0 | 4 | | | - | - | - | | | 4 | 2 | 2 | | |
| Buccal mucosa | 41 | 15 | 26 | | | 1 | 0 | 1 | | | 3 | 2 | 1 | | |
| Gingivae | 6 | 2 | 4 | | | - | - | - | | | 4 | 2 | 2 | | |
| Hard palate | 2 | 0 | 2 | | | 1 | 0 | 1 | | | - | - | - | | |
| Upper lip | 2 | 0 | 2 | | | - | - | - | | | - | - | - | | |
| Skin | 1 | 0 | 1 | | | - | - | - | | | - | - | - | | |
| Total | 84 | 28 | 56 | *df=9* | *CV=0.312*$^{\alpha}$ | 12 | 2 | 10 | *df=3* | *CV=0.418*$^{\alpha}$ | 80 | 24 | 56 | *df=6* | *CV=0.330*$^{\alpha}$ |
| Tumour size | | | | | | | | | | | | | | 5.625 | 0.131 |
| T1 | | | | | | | | | | | 41 | 9 | 32 | | |
| T2 | | | | | | | | | | | 22 | 6 | 16 | | |
| T3 | | | | | | | | | | | 13 | 7 | 6 | | |
| T4 | | | | | | | | | | | 4 | 2 | 2 | | |
| Total | | | | | | | | | | | 80 | 24 | 56 | *df=3* | *CV=0.265*$^{\alpha}$ |
| Regional lymph nodes | | | | | | | | | | | | | | 5.075 | 0.079 |
| N0 | | | | | | | | | | | 67 | 17 | 50 | | |
| N1 | | | | | | | | | | | 7 | 3 | 4 | | |
| N2 | | | | | | | | | | | 6 | 4 | 2 | | |
| Total | | | | | | | | | | | 80 | 24 | 56 | *df=2* | *CV=0.252*$^{\alpha}$ |
| Differentiation | | | | | | | | | | | | | | 4.269 | 0.234 |
| Basal | | | | | | | | | | | 2 | 1 | 1 | | |
| Poor | | | | | | | | | | | 11 | 6 | 5 | | |
| Moderate | | | | | | | | | | | 58 | 15 | 43 | | |
| Well | | | | | | | | | | | 9 | 2 | 7 | | |
| Total | | | | | | | | | | | 80 | 24 | 56 | *df=3* | *CV=0.231*$^{\alpha}$ |

Note: $^{\alpha}$ generated from Cramer's V Test

## Appendix 47

Table A: Crosstabs with Chi-square and Phi Analyses of HR α-HPV among clinicopathological parameters

| Variable | Benign (*N*=84) | | | | | Dysplasia (*N*=12) | | | | | Carcinoma (*N*=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HR α-HPV | | | | | HR α-HPV | | | | | HR α-HPV | | | |
| | *N* | positive | Negative | $\chi^2$ | *p value* | *N* | positive | negative | $\chi^2$ | *p value* | *n* | Positive | negative | $\chi^2$ | *p value* |
| Gender | | | | 0.086 | 0.769 | | | | 0.779 | 0.377[α] | | | | 3.570 | 0.059 |
| Female | 45 | 8 | 37 | | | 5 | 0 | 5 | | | 49 | 11 | 38 | | |
| Male | 39 | 6 | 33 | | | 7 | 1 | 6 | | | 31 | 2 | 29 | | |
| Total | 84 | 14 | 70 | *df=1* | *Φ=(0.032)* | 12 | 1 | 11 | *df=1* | *Φ=0.255* | 80 | 13 | 67 | *df=1* | *Φ=(0.211)* |
| Age group | | | | 4.887* | 0.027 | | | | 5.455* | 0.020[α] | | | | 5.389* | 0.020 |
| 16-57y | 52 | 5 | 47 | | | 2 | 1 | 1 | | | 38 | 10 | 28 | | |
| 58-99y | 32 | 9 | 23 | | | 10 | 0 | 10 | | | 42 | 3 | 39 | | |
| Total | 84 | 14 | 70 | *df=1* | *Φ=(0.241)* | 12 | 1 | 11 | *df=1* | *Φ=(0.674)* | 80 | 13 | 67 | *df=1* | *Φ=(0.260)* |
| Disease outcome | | | | | | | | | | | | | | 5.075* | 0.024 |
| Alive | | | | | | | | | | | 35 | 2 | 33 | | |
| Dead | | | | | | | | | | | 45 | 11 | 34 | | |
| Total | | | | | | | | | | | 80 | 13 | 67 | *df=1* | *Φ=(0.252)* |
| Disease status | | | | N/A | N/A | | | | N/A | N/A | | | | 1.035 | 0.309[α] |
| No Recurrence | 84 | 14 | 70 | | | 12 | 1 | 11 | | | 75 | 13 | 62 | | |
| Recurrence | 0 | 0 | 0 | | | 0 | 0 | 0 | | | 5 | 0 | 5 | | |
| Total | 84 | 14 | 70 | | | 12 | 1 | 11 | | | 80 | 13 | 67 | *df=1* | *Φ=(0.114)* |
| DNA purity | | | | 4.222* | 0.040 | | | | 0.364 | 0.546[α] | | | | 8.826** | 0.003[α] |
| < 1.043 | 45 | 11 | 34 | | | 9 | 1 | 8 | | | 29 | 0 | 29 | | |
| 1.043 and above | 39 | 3 | 36 | | | 3 | 0 | 3 | | | 51 | 13 | 38 | | |
| Total | 84 | 14 | 70 | *df=1* | *Φ=(0.224)* | 12 | 1 | 11 | *df=1* | *Φ=(0.174)* | 80 | 13 | 67 | *df=1* | *Φ=(0.332)* |
| DNA yield | | | | 4.222* | 0.040 | | | | 1.091 | 0.296[α] | | | | 0.827 | 0.363 |
| < 0.074 μg/μl | 45 | 4 | 41 | | | 6 | 0 | 6 | | | 40 | 8 | 32 | | |
| 0.074 and above | 39 | 10 | 29 | | | 6 | 1 | 5 | | | 40 | 5 | 35 | | |
| Total | 84 | 14 | 70 | *df=1* | *Φ=(0.224)* | 12 | 1 | 11 | *df=1* | *Φ=0.302* | 80 | 13 | 67 | *df=1* | *Φ=(0.102)* |

Note: [α] generated from Fisher's Exact Test; *Φ* in parentheses to denote negative value; * significant at the 0.05 level; ** significant at the 0.01 level

## Appendix 47 (continued)

Table B: Crosstabs with Chi-square and Cramer's V analyses of HR α-HPV among clinicopathological parameters

| Variable | Benign (*N*=84) | | | | | Dysplasia (*N*=12) | | | | | Carcinoma (*N*=80) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | HR α-HPV | | | | | HR α-HPV | | | | | HR α-HPV | | | |
| | *N* | positive | Negative | $\chi^2$ | *p value* | *N* | positive | negative | $\chi^2$ | *p value* | *N* | positive | negative | $\chi^2$ | *p value* |
| Anatomic site | | | | 7.810 | 0.553 | | | | 0.545 | 0.909 | | | | 6.637 | 0.356 |
| Tongue | 21 | 2 | 19 | | | - | - | - | | | 20 | 1 | 19 | | |
| Floor of the mouth | 3 | 0 | 3 | | | 8 | 1 | 7 | | | 12 | 2 | 10 | | |
| Soft palate | 3 | 0 | 3 | | | 2 | 0 | 2 | | | 34 | 9 | 25 | | |
| Retromolar pad | 1 | 0 | 1 | | | - | - | - | | | 3 | 0 | 3 | | |
| Lower lip | 4 | 2 | 2 | | | - | - | - | | | 4 | 0 | 4 | | |
| Buccal mucosa | 41 | 8 | 33 | | | 1 | 0 | 1 | | | 3 | 0 | 3 | | |
| Gingivae | 6 | 1 | 5 | | | - | - | - | | | 4 | 1 | 3 | | |
| Hard palate | 2 | 0 | 2 | | | 1 | 0 | 1 | | | - | - | - | | |
| Upper lip | 2 | 1 | 1 | | | - | - | - | | | - | - | - | | |
| Skin | 1 | 0 | 1 | | | - | - | - | | | - | - | - | | |
| Total | 84 | 14 | 70 | *df=9* | *CV=0.305*$^{\alpha}$ | 12 | 1 | 11 | *df=3* | *CV=0.213*$^{\alpha}$ | 80 | 13 | 67 | *df=6* | *CV=0.288*$^{\alpha}$ |
| Tumour size | | | | | | | | | | | | | | 8.334* | 0.040 |
| T1 | | | | | | | | | | | 41 | 11 | 30 | | |
| T2 | | | | | | | | | | | 22 | 1 | 21 | | |
| T3 | | | | | | | | | | | 13 | 0 | 13 | | |
| T4 | | | | | | | | | | | 4 | 1 | 3 | | |
| Total | | | | | | | | | | | 80 | 13 | 67 | *df=3* | *CV=0.323*$^{\alpha}$ |
| Regional lymph nodes | | | | | | | | | | | | | | 0.022 | 0.989 |
| N0 | | | | | | | | | | | 67 | 11 | 56 | | |
| N1 | | | | | | | | | | | 7 | 1 | 6 | | |
| N2 | | | | | | | | | | | 6 | 1 | 5 | | |
| Total | | | | | | | | | | | 80 | 13 | 67 | *df=2* | *CV=0.017*$^{\alpha}$ |
| Differentiation | | | | | | | | | | | | | | 3.389 | 0.335 |
| Basal | | | | | | | | | | | 2 | 0 | 2 | | |
| Poor | | | | | | | | | | | 11 | 1 | 10 | | |
| Moderate | | | | | | | | | | | 58 | 12 | 46 | | |
| Well | | | | | | | | | | | 9 | 0 | 9 | | |
| Total | | | | | | | | | | | 80 | 13 | 67 | *df=3* | *CV=0.206*$^{\alpha}$ |

Note: $^{\alpha}$ generated from Cramer's V Test; * significant at the 0.05 level; ** significant at the 0.01 level

# Appendix 48

## Table A: Pearson's correlation between clinical parameters and HPV status in benign

| Clinical parameters | HPV positivity ($N$=84) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* |
| Gender | -0.046 | 0.677 | -0.027 | 0.805 | 0.051 | 0.647 | -0.032 | 0.772 |
| Age group | 0.030 | 0.784 | 0.086 | 0.434 | -0.035 | 0.754 | 0.241* | 0.027 |
| Anatomic site | -0.017 | 0.878 | -0.069 | 0.533 | -0.048 | 0.662 | 0.068 | 0.537 |
| DNA purity | -0.046 | 0.677 | -0.027 | 0.805 | 0.101 | 0.359 | -0.224* | 0.040 |
| DNA yield | 0.240* | 0.028 | 0.212 | 0.053 | 0.101 | 0.359 | 0.224* | 0.040 |

Note: * significant at the 0.05 level

## Table B: Spearman's correlation between clinical parameters and HPV status in benign

| Clinical parameters | HPV positivity ($N$=84) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* |
| Gender | -0.046 | 0.677 | -0.027 | 0.805 | 0.051 | 0.647 | -0.032 | 0.772 |
| Age group | 0.030 | 0.784 | 0.086 | 0.434 | -0.035 | 0.754 | 0.241* | 0.027 |
| Anatomic site | -0.050 | 0.654 | -0.093 | 0.398 | -0.055 | 0.621 | 0.079 | 0.474 |
| DNA purity | -0.046 | 0.677 | -0.027 | 0.805 | 0.101 | 0.359 | -0.224* | 0.040 |
| DNA yield | 0.240* | 0.028 | 0.212 | 0.053 | 0.101 | 0.359 | 0.224* | 0.040 |

Note: * significant at the 0.05 level

# Appendix 49

## Table A: Pearson's correlation between clinical parameters and HPV status in dysplasia

| Clinical parameters | HPV positivity (*N*=12) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* |
| Gender | 0.371 | 0.235 | 0.488 | 0.108 | 0.378 | 0.226 | 0.255 | 0.424 |
| Age group | -0.529 | 0.077 | -0.258 | 0.418 | 0.200 | 0.533 | -0.674* | 0.016 |
| Anatomic site | -0.123 | 0.702 | -0.192 | 0.551 | -0.327 | 0.300 | -0.140 | 0.664 |
| DNA purity | -0.098 | 0.763 | 0.111 | 0.731 | 0.258 | 0.418 | -0.174 | 0.588 |
| DNA yield | 0.169 | 0.599 | 0.192 | 0.549 | 0.00 | 1.000 | 0.302 | 0.341 |

Note: * significant at the 0.05 level

## Table B: Spearman's correlation between clinical parameters and HPV status in dysplasia

| Clinical parameters | HPV positivity (*N*=12) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* |
| Gender | 0.371 | 0.235 | 0.488 | 0.108 | 0.378 | 0.226 | 0.255 | 0.424 |
| Age group | -0.529 | 0.077 | -0.258 | 0.418 | 0.200 | 0.533 | -0.674* | 0.016 |
| Anatomic site | -0.204 | 0.524 | -0.200 | 0.534 | 0.309 | 0.328 | 0.104 | 0.747 |
| DNA purity | -0.098 | 0.763 | 0.111 | 0.731 | 0.258 | 0.418 | -0.174 | 0.588 |
| DNA yield | 0.169 | 0.599 | 0.192 | 0.549 | 0.000 | 1.000 | 0.302 | 0.341 |

Note: * significant at the 0.05 level

# Appendix 50

## Table A: Pearson's correlation between clinical parameters and HPV status in carcinoma

| Clinical parameters | HPV positivity ($N$=80) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* |
| Gender | -0.066 | 0.564 | -0.069 | 0.544 | 0.095 | 0.401 | -0.211 | 0.060 |
| Age group | -0.053 | 0.643 | -0.021 | 0.851 | 0.186 | 0.099 | -0.260* | 0.020 |
| Anatomic site | -0.225* | 0.044 | -0.279* | 0.012 | 0.182 | 0.106 | 0.151 | 0.180 |
| Disease outcome | 0.019 | 0.868 | 0.009 | 0.934 | -0.192 | 0.087 | 0252* | 0.024 |
| Disease status | -0.065 | 0.569 | -0.032 | 0.776 | 0.056 | 0.620 | -0.114 | 0.315 |
| Tumour size | 0.097 | 0.391 | 0.063 | 0.581 | 0.242* | 0.030 | -0.216 | 0.054 |
| Regional lymph nodes | 0.175 | 0.120 | 0.227* | 0.043 | 0.251* | 0.025 | -0.005 | 0.964 |
| Histological differentiation | -0.250* | 0.026 | -0.180 | 0.109 | -0.195 | 0.083 | -0.001 | 0.990 |
| DNA purity | 0.116 | 0.306 | 0.074 | 0.516 | -0.187 | 0.096 | 0.332** | 0.003 |
| DNA yield | -0.100 | 0.377 | -0.025 | 0.825 | 0.055 | 0.631 | -0.102 | 0.370 |

Note: ** significant at the 0.01 level. * significant at the 0.05 level

## Table B: Spearman's correlation between clinical parameters and HPV status in carcinoma

| Clinical parameters | HPV positivity ($N$=80) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HPV | | α-HPV | | LR α-HPV | | HR α-HPV | |
| | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* |
| Gender | -0.066 | 0.564 | -0.069 | 0.544 | 0.095 | 0.401 | -0.211 | 0.060 |
| Age group | -0.053 | 0.643 | -0.021 | 0.851 | 0.186 | 0.099 | -0.260* | 0.020 |
| Anatomic site | -0.228* | 0.042 | -0.283* | 0.011 | -0.195 | 0.083 | 0.140 | 0.214 |
| Disease outcome | 0.019 | 0.868 | 0.009 | 0.934 | -0.192 | 0.087 | 0252* | 0.024 |
| Disease status | -0.065 | 0.569 | -0.032 | 0.776 | 0.056 | 0.620 | -0.114 | 0.315 |
| Tumour size | 0.055 | 0.628 | 0.017 | 0.880 | 0.231* | 0.040 | -0.263* | 0.018 |
| Regional lymph nodes | 0.156 | 0.166 | 0.212 | 0.060 | 0.238* | 0.034 | -0.009 | 0.936 |
| Histological differentiation | -0.277* | 0.013 | -0.196 | 0.082 | -0.200 | 0.075 | -0.016 | 0.889 |
| DNA purity | 0.116 | 0.306 | 0.074 | 0.516 | -0.187 | 0.096 | 0.332** | 0.003 |
| DNA yield | -0.100 | 0.377 | -0.025 | 0.825 | 0.055 | 0.631 | -0.102 | 0.370 |

Note: ** significant at the 0.01 level. * significant at the 0.05 level

# Appendix 51

Table A: Pearson's correlation between clinical parameters and p16 status

| Clinical parameters | p16 positivity | | | | | |
|---|---|---|---|---|---|---|
| | Benign (*N*=82) | | Dysplasia (*N*=12) | | Carcinoma (*N*=80) | |
| | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* |
| Gender | -0.097 | 0.388 | 0.478 | 0.116 | 0.400** | 0.000 |
| Age group | 0.030 | 0.786 | -0.316 | 0.317 | 0.491** | 0.000 |
| Anatomic site | -0.103 | 0.355 | 0.047 | 0.885 | 0.202 | 0.072 |
| Disease outcome | - | - | - | - | 0.149 | 0.187 |
| Disease status | - | - | - | - | -0.451** | 0.000 |
| Tumour size | - | - | - | - | 0.416** | 0.000 |
| Regional lymph nodes | - | - | - | - | 0.206 | 0.066 |
| Histological grade | - | - | - | - | -0.025 | 0.829 |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

Table B: Spearman's correlation between clinical parameters and p16 status

| Clinical parameters | p16 positivity | | | | | |
|---|---|---|---|---|---|---|
| | Benign (*N*=84) | | Dysplasia (*N*=12) | | Carcinoma (*N*=80) | |
| | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* |
| Gender | -0.097 | 0.388 | 0.478 | 0.116 | 0.334** | 0.002 |
| Age group | 0.030 | 0.786 | -0.316 | 0.317 | 0.463** | 0.000 |
| Anatomic site | -0.121 | 0.281 | -0.031 | 0.925 | 0.130 | 0.252 |
| Disease outcome | - | - | - | - | 0.129 | 0.254 |
| Disease status | - | - | - | - | -0.441** | 0.000 |
| Tumour size | - | - | - | - | 0.461** | 0.000 |
| Regional lymph nodes | - | - | - | - | 0.219 | 0.051 |
| Histological grade | - | - | - | - | 0.016 | 0.891 |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

## Appendix 52

Table A: Pearson's correlation between clinical parameters and p16 staining status

| Clinical parameters | p16 staining intensity | | | | | |
|---|---|---|---|---|---|---|
| | Benign (*N*=82) | | Dysplasia (*N*=12) | | Carcinoma (*N*=80) | |
| | *r* | *Sig.* | *r* | *Sig.* | *r* | *Sig.* |
| Gender | -0.095 | 0.395 | 0.439 | 0.154 | 0.359** | 0.001 |
| Age group | -0.001 | 0.994 | -0.073 | 0.823 | 0.524** | 0.000 |
| Anatomic site | -0.057 | 0.609 | 0.237 | 0.459 | 0.242* | 0.031 |
| Disease outcome | - | - | - | - | 0.217 | 0.053 |
| Disease status | - | - | - | - | -0.448** | 0.000 |
| Tumour size | - | - | - | - | 0.471** | 0.000 |
| Regional lymph nodes | - | - | - | - | 0.059 | 0.602 |
| Histological grade | - | - | - | - | -0.079 | 0.489 |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

Table B: Spearman's correlation between clinical parameters and p16 status

| Clinical parameters | p16 staining intensity | | | | | |
|---|---|---|---|---|---|---|
| | Benign (*N*=82) | | Dysplasia (*N*=12) | | Carcinoma (*N*=80) | |
| | *rho* | *Sig.* | *rho* | *Sig.* | *rho* | *Sig.* |
| Gender | -0.098 | 0.383 | 0.427 | 0.167 | 0.359** | 0.001 |
| Age group | 0.007 | 0.947 | -0.035 | 0.913 | 0.520** | 0.000 |
| Anatomic site | -0.089 | 0.426 | 0.164 | 0.611 | 0.190 | 0.092 |
| Disease outcome | - | - | - | - | 0.222* | 0.048 |
| Disease status | - | - | - | - | -0.440** | 0.000 |
| Tumour size | - | - | - | - | 0.544** | 0.000 |
| Regional lymph nodes | - | - | - | - | 0.061 | 0.594 |
| Histological grade | - | - | - | - | -0.103 | 0.364 |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

## Appendix 53

Table A: Crosstabs with Chi-square test of HPV status among clinicopathological parameters

| Parameters | *N* | Overall HPV | | $\chi^2$ | *p value* | *N* | α-HPV | | $\chi^2$ | *p value* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | negative | positive | | | | negative | positive | | |
| Gender | | | | 0.028 | 0.867 | | | | 0.024 | 0.877 |
| Female | 76 | 31 | 45 | | | 76 | 37 | 39 | | |
| Male | 64 | 27 | 37 | | | 64 | 32 | 32 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |
| Age group | | | | 0.440 | 0.507 | | | | 0.000 | 0.990 |
| 36-64y | 75 | 33 | 42 | | | 75 | 37 | 38 | | |
| 65-99y | 65 | 25 | 40 | | | 65 | 32 | 33 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |
| Disease outcome | | | | 0.251 | 0.616 | | | | 0.001 | 0.979 |
| Alive | 81 | 35 | 46 | | | 81 | 40 | 41 | | |
| Dead | 59 | 23 | 36 | | | 59 | 29 | 30 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |
| Disease status | | | | 0.126 | 0.722 | | | | 0.798 | 0.372 |
| No Recurrence | 129 | 54 | 75 | | | 129 | 65 | 64 | | |
| Recurrence | 11 | 4 | 7 | | | 11 | 4 | 7 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

**Appendix 53 (continued)**

Table A: Crosstabs with Chi-square test of HPV status among clinicopathological parameters

| Parameters | *N* | HR- HPV | | $\chi^2$ | *p value* | *N* | LR-HPV | | $\chi^2$ | *p value* | *N* | HPV16 | | $\chi^2$ | *p value* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | negative | positive | | | | negative | positive | | | | negative | positive | | |
| Gender | | | | 1.789 | 0.181 | | | | 0.262 | 0.609 | | | | 6.142* | 0.013 |
| Female | 76 | 60 | 16 | | | 76 | 46 | 30 | | | 76 | 62 | 14 | | |
| Male | 64 | 56 | 8 | | | 64 | 36 | 28 | | | 64 | 61 | 3 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |
| Age group | | | | 1.997 | 0.158 | | | | 4.363* | 0.037 | | | | 2.253 | 0.133 |
| 36-64y | 75 | 59 | 16 | | | 75 | 50 | 25 | | | 75 | 63 | 12 | | |
| 65-99y | 65 | 57 | 8 | | | 65 | 32 | 33 | | | 65 | 60 | 5 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |
| Disease outcome | | | | 5.394* | 0.020 | | | | 0.789 | 0.374 | | | | 7.323** | 0.007 |
| Alive | 81 | 62 | 19 | | | 81 | 50 | 31 | | | 81 | 66 | 15 | | |
| Dead | 59 | 54 | 5 | | | 59 | 32 | 27 | | | 59 | 57 | 2 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |
| Disease status | | | | 0.009 | 0.924 | | | | 2.426 | 0.119 | | | | 0.408 | 0.523 |
| No Recurrence | 129 | 107 | 22 | | | 129 | 78 | 51 | | | 129 | 114 | 15 | | |
| Recurrence | 11 | 9 | 2 | | | 11 | 4 | 7 | | | 11 | 9 | 2 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

**Appendix 53 (continued)**

Table B: Crosstabs with Chi-square and Cramer's V tests of p16 status among clinicopathological parameters

| Parameters | *N* | p16 positivity | | $\chi^2$ | *p value* |
|---|---|---|---|---|---|
| | | negative | positive | | |
| Gender | | | | 8.022** | 0.005 |
| Female | 76 | 25 | 51 | | |
| Male | 64 | 8 | 56 | | |
| Total | 140 | 33 | 107 | | |
| Age group | | | | 8.545** | 0.003 |
| 36-64y | 75 | 25 | 50 | | |
| 65-99y | 65 | 8 | 57 | | |
| Total | 140 | 33 | 107 | | |
| Disease outcome | | | | 15.960** | 0.000 |
| Alive | 81 | 29 | 52 | | |
| Dead | 59 | 4 | 55 | | |
| Total | 140 | 33 | 107 | | |
| Disease status | | | | 1.389 | 0.238 |
| No Recurrence | 129 | 32 | 97 | | |
| Recurrence | 11 | 1 | 10 | | |
| Total | 140 | 33 | 107 | | |

| Parameters | *N* | p16 staining intensity | | | | Cramer's V value | *p value* |
|---|---|---|---|---|---|---|---|
| | | negative | 1+ | 2+ | 3+ | | |
| Gender | | | | | | 0.258* | 0.026 |
| Female | 76 | 25 | 22 | 25 | 4 | | |
| Male | 64 | 8 | 19 | 30 | 7 | | |
| Total | 140 | 33 | 41 | 55 | 11 | | |
| Age group | | | | | | 0.297** | 0.006 |
| 36-64y | 75 | 25 | 24 | 22 | 4 | | |
| 65-99y | 65 | 8 | 17 | 33 | 7 | | |
| Total | 140 | 33 | 41 | 55 | 11 | | |
| Disease outcome | | | | | | 0.403** | 0.000 |
| Alive | 81 | 29 | 26 | 23 | 3 | | |
| Dead | 59 | 4 | 15 | 32 | 8 | | |
| Total | 140 | 33 | 41 | 55 | 11 | | |
| Disease status | | | | | | 0.142 | 0.421 |
| No Recurrence | 129 | 32 | 38 | 50 | 9 | | |
| Recurrence | 11 | 1 | 3 | 5 | 2 | | |
| Total | 140 | 33 | 41 | 55 | 11 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

## Appendix 53 (continued)

Table C: Crosstabs with Cramer's V test of HPV status among clinicopathological parameters

| Parameters | *N* | Overall HPV | | Cramer's V value | *p value* | *N* | α-HPV | | Cramer's V value | *p value* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | negative | positive | | | | negative | Positive | | |
| Anatomic site | | | | 0.191 | 0.885 | | | | 0.224 | 0.721 |
| Buccal mucosa | 4 | 1 | 3 | | | 4 | 1 | 3 | | |
| Floor of the mouth | 22 | 10 | 12 | | | 22 | 11 | 11 | | |
| Alveolus | 4 | 1 | 3 | | | 4 | 1 | 3 | | |
| Gingivae | 4 | 1 | 3 | | | 4 | 1 | 3 | | |
| Soft palate | 37 | 15 | 22 | | | 37 | 19 | 18 | | |
| Pharynx | 1 | - | 1 | | | 1 | - | 1 | | |
| Lower lip | 5 | 2 | 3 | | | 5 | 2 | 3 | | |
| Retromolar pad | 12 | 4 | 8 | | | 12 | 5 | 7 | | |
| Supraglottis | 1 | - | 1 | | | 1 | 1 | - | | |
| Tongue | 49 | 24 | 25 | | | 49 | 27 | 22 | | |
| Tonsil | 1 | - | 1 | | | 1 | 1 | - | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |
| Tumour size | | | | 0.225 | 0.058 | | | | 0.219 | 0.150 |
| Negative | 2 | - | 2 | | | 2 | 1 | 1 | | |
| $T_1$ (<2 cm) | 65 | 30 | 35 | | | 65 | 33 | 32 | | |
| $T_2$ (2-4 cm) | 41 | 21 | 20 | | | 41 | 24 | 17 | | |
| $T_3$ (>4 cm) | 14 | 4 | 10 | | | 14 | 7 | 7 | | |
| $T_4$ (>4 cm) | 18 | 3 | 15 | | | 18 | 4 | 14 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |
| Regional Lymph Nodes | | | | 0.187 | 0.178 | | | | 0.190 | 0.166 |
| Negative | 71 | 35 | 36 | | | 71 | 41 | 30 | | |
| $N_0$ | 35 | 13 | 22 | | | 35 | 14 | 21 | | |
| $N_1$ | 16 | 6 | 10 | | | 16 | 8 | 8 | | |
| $N_2$ | 18 | 4 | 14 | | | 18 | 6 | 12 | | |
| Total | 140 | 58 | 82 | | | 140 | 69 | 71 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

**Appendix 53 (continued)**

Table C: Crosstabs with Cramer's V test of HPV status among clinicopathological parameters

| Parameters | *N* | HR-HPV | | Cramer's V value | *p value* | *N* | LR-HPV | | Cramer's V value | *p value* | *N* | HPV16 | | Cramer's V value | *p value* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | negative | positive | | | | negative | Positive | | | | negative | Positive | | |
| Anatomic site | | | | 0.204 | 0.828 | | | | 0.334 | 0.111 | | | | 0.312 | 0.192 |
| Buccal mucosa | 4 | 4 | - | | | 4 | 1 | 3 | | | 4 | 4 | - | | |
| Floor of the mouth | 22 | 19 | 3 | | | 22 | 12 | 10 | | | 22 | 21 | 1 | | |
| Alveolus | 4 | 3 | 1 | | | 4 | 1 | 3 | | | 4 | 3 | 1 | | |
| Gingivae | 4 | 3 | 1 | | | 4 | 2 | 2 | | | 4 | 4 | - | | |
| Soft palate | 37 | 27 | 10 | | | 37 | 29 | 8 | | | 37 | 27 | 10 | | |
| Pharynx | 1 | 1 | - | | | 1 | - | 1 | | | 1 | 1 | - | | |
| Lower lip | 5 | 5 | - | | | 5 | 2 | 3 | | | 5 | 5 | - | | |
| Retromolar pad | 12 | 10 | 2 | | | 12 | 5 | 7 | | | 12 | 10 | 2 | | |
| Supraglottis | 1 | 1 | - | | | 1 | - | 1 | | | 1 | 1 | - | | |
| Tongue | 49 | 42 | 7 | | | 49 | 29 | 20 | | | 49 | 46 | 3 | | |
| Tonsil | 1 | 1 | - | | | 1 | 1 | - | | | 1 | 1 | - | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |
| Tumour size | | | | 0.248 | 0.072 | | | | 0.301* | 0.013 | | | | 0.272* | 0.035 |
| Negative | 2 | 2 | - | | | 2 | 1 | 1 | | | 2 | 2 | - | | |
| $T_1$ (<2 cm) | 65 | 48 | 17 | | | 65 | 43 | 22 | | | 65 | 51 | 14 | | |
| $T_2$ (2-4 cm) | 41 | 37 | 4 | | | 41 | 27 | 14 | | | 41 | 39 | 2 | | |
| $T_3$ (>4 cm) | 14 | 14 | - | | | 14 | 7 | 7 | | | 14 | 14 | - | | |
| $T_4$ (>4 cm) | 18 | 15 | 3 | | | 18 | 4 | 14 | | | 18 | 17 | 1 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |
| Regional Lymph Nodes | | | | 0.070 | 0.875 | | | | 0.330** | 0.002 | | | | 0.137 | 0.452 |
| Negative | 71 | 59 | 12 | | | 71 | 52 | 19 | | | 71 | 61 | 10 | | |
| $N_0$ | 35 | 28 | 7 | | | 35 | 17 | 18 | | | 35 | 30 | 5 | | |
| $N_1$ | 16 | 13 | 3 | | | 16 | 8 | 8 | | | 16 | 16 | - | | |
| $N_2$ | 18 | 16 | 2 | | | 18 | 5 | 13 | | | 18 | 16 | 2 | | |
| Total | 140 | 116 | 24 | | | 140 | 82 | 58 | | | 140 | 123 | 17 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

**Appendix 53 (continued)**

Table D: Crosstabs with Cramer's V test of p16 status among clinicopathological parameters

| Parameters | *N* | p16 positivity | | Cramer's V value | *p value* | *N* | p16 staining intensity | | | | Cramer's V value | *p value* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | negative | positive | | | | Negative | 1+ | 2+ | 3+ | | |
| Anatomic site | | | | 0.409** | 0.009 | | | | | | 0.300 | 0.154 |
| Buccal mucosa | 4 | - | 4 | | | 4 | - | 1 | 3 | - | | |
| Floor of the mouth | 22 | 4 | 18 | | | 22 | 4 | 8 | 10 | - | | |
| Alveolus | 4 | 2 | 2 | | | 4 | 2 | 2 | - | - | | |
| Gingivae | 4 | 1 | 3 | | | 4 | 1 | 1 | 2 | - | | |
| Soft palate | 37 | 18 | 19 | | | 37 | 18 | 7 | 9 | 3 | | |
| Pharynx | 1 | - | 1 | | | 1 | - | - | 1 | - | | |
| Lower lip | 5 | - | 5 | | | 5 | - | 2 | 3 | - | | |
| Retromolar pad | 12 | 3 | 9 | | | 12 | 3 | 2 | 6 | 1 | | |
| Supraglottis | 1 | - | 1 | | | 1 | - | - | 1 | - | | |
| Tongue | 49 | 5 | 44 | | | 49 | 5 | 18 | 19 | 7 | | |
| Tonsil | 1 | - | 1 | | | 1 | - | - | 1 | - | | |
| Total | 140 | 33 | 107 | | | 140 | 33 | 41 | 55 | 11 | | |
| Tumour size | | | | 0.373** | 0.001 | | | | | | 0.309** | 0.000 |
| Negative | 2 | - | 2 | | | 2 | - | 1 | 1 | - | | |
| $T_1$ (<2 cm) | 65 | 26 | 39 | | | 65 | 26 | 21 | 16 | 2 | | |
| $T_2$ (2-4 cm) | 41 | 4 | 37 | | | 41 | 4 | 13 | 17 | 7 | | |
| $T_3$ (>4 cm) | 14 | - | 14 | | | 14 | - | - | 12 | 2 | | |
| $T_4$ (>4 cm) | 18 | 3 | 15 | | | 18 | 3 | 6 | 9 | - | | |
| Total | 140 | 33 | 107 | | | 140 | 33 | 41 | 55 | 11 | | |
| Regional Lymph Nodes | | | | 0.131 | 0.492 | | | | | | 0.139 | 0.526 |
| Negative | 71 | 20 | 51 | | | 71 | 20 | 16 | 27 | 8 | | |
| $N_0$ | 35 | 8 | 27 | | | 35 | 8 | 11 | 14 | 2 | | |
| $N_1$ | 16 | 2 | 14 | | | 16 | 2 | 6 | 8 | - | | |
| $N_2$ | 18 | 3 | 15 | | | 18 | 3 | 8 | 6 | 1 | | |
| Total | 140 | 33 | 107 | | | 140 | 33 | 41 | 55 | 11 | | |

Note: * significant at the 0.05 level; ** significant at the 0.01 level

## Appendix 54: The overall purity and yield of the extracted DNA

| Parameters | $N$ | Mean $\pm$ SD$^{\alpha}$ | |
|---|---|---|---|
| | | DNA purity ($A_{260}/A_{280}$) | DNA yield (µg/µl) |
| Gender ($N$ = 183) | | | |
| Female | 102 | 1.394 $\pm$ 0.475 | 0.269 $\pm$ 0.434 |
| Male | 81 | 1.292 $\pm$ 0.435 | 0.291 $\pm$ 0.427 |
| Age group$^{\beta}$ ($N$ = 183) | | | |
| 16-57y | 96 | 1.429 $\pm$ 0.479 | 0.167 $\pm$ 0.309 |
| 58-99y | 87 | 1.261 $\pm$ 0.422 | 0.402 $\pm$ 0.507 |
| Anatomic site ($N$ = 183) | | | |
| Buccal mucosa | 46 | 1.202 $\pm$ 0.416 | 0.285 $\pm$ 0.432 |
| Floor of the mouth | 23 | 1.358 $\pm$ 0.489 | 0.403 $\pm$ 0.550 |
| Gingivae | 10 | 1.137 $\pm$ 0.367 | 0.704 $\pm$ 0.547 |
| Hard palate | 3 | 1.149 $\pm$ 0.412 | 0.079 $\pm$ 0.025 |
| Soft palate | 39 | 1.604 $\pm$ 0.416 | 0.127 $\pm$ 0.279 |
| Upper lip | 2 | 1.815 $\pm$ 0.284 | 0.110 $\pm$ 0.027 |
| Lower lip | 9 | 1.143 $\pm$ 0.350 | 0.406 $\pm$ 0.474 |
| Retromolar pad | 5 | 1.549 $\pm$ 0.297$^{\varepsilon}$ | 0.076 $\pm$ 0.031$^{\varepsilon}$ |
| Skin | 1 | 1.784 | 0.066 |
| Tongue | 44 | 1.314 $\pm$ 0.489 | 0.273 $\pm$ 0.418 |
| Tonsil | 1 | 1.759 | 0.143 |
| Pathology ($N$ = 183) | | | |
| Normal | 7 | 1.172 $\pm$ 0.406 | 0.183 $\pm$ 0.113 |
| Benign | 84 | 1.317 $\pm$ 0.470 | 0.212 $\pm$ 0.358 |
| Dysplasia | 12 | 1.064 $\pm$ 0.209 | 0.389 $\pm$ 0.569 |
| Carcinoma | 80 | 1.441 $\pm$ 0.458 | 0.341 $\pm$ 0.484 |
| Disease status ($N$ = 183) | | | |
| No recurrence | 171 | 1.345 $\pm$ 0.456 | 0.278 + 0.430 |
| Recurrence | 5 | 1.491 $\pm$ 0.593 | 0.297 + 0.465 |
| Disease outcome ($N$ = 81) | | | |
| Alive | 45 | 1.232 $\pm$ 0.432 | 0.563 $\pm$ 0.557 |
| Dead | 35 | 1.597 $\pm$ 0.417 | 0.162 $\pm$ 0.317 |
| Tumour size ($N$ = 80) | | | |
| $T_1$ (<2 cm) | 41 | 1.596 $\pm$ 0.437 | 0.228 $\pm$ 0.407 |
| $T_2$ (2-4 cm) | 22 | 1.296 $\pm$ 0.425 | 0.402 $\pm$ 0.523 |
| $T_3$ (>4 cm) | 13 | 1.124 $\pm$ 0.210 | 0.504 $\pm$ 0.555 |
| $T_4$ (>4 cm) and invades adjacent structures | 4 | 1.672 $\pm$ 0.742 | 0.638 $\pm$ 0.617 |
| Regional Lymph Nodes ($N$ = 80) | | | |
| $N_0$ | 67 | 1.483 $\pm$ 0.444 | 0.216 $\pm$ 0.374 |
| $N_1$ | 7 | 1.023 $\pm$ 0.011$^{\varepsilon}$ | 1.112 $\pm$ 0.399 |
| $N_2$ | 6 | 1.454 $\pm$ 0.667 | 0.835 $\pm$ 0.569 |
| Histological grade ($N$ = 80) | | | |
| Well differentiated | 9 | 1.251 $\pm$ 0.352 | 0.423 + 0.548 |
| Moderately differentiated | 58 | 1.502 $\pm$ 0.464 | 0.261 + 0.422 |
| Poorly differentiated | 11 | 1.171 $\pm$ 0.348 | 0.739 + 0.605 |
| Basal | 2 | 2.015 $\pm$ 0.371 | 0.107 + 0.026 |

*Note: N, number of patients; SD, standard deviation; $^{\alpha}$ - No SD value was calculated for single case; $^{\beta}$ - Age grouping was based on overall median age; $^{\varepsilon}$ - Normal distribution data is assumed*

## PUBLICATIONS

1. A.T. Sailan, K. Purdie, M.N.C. de Koning, W.G.V. Quint, P.A. Mossey J. Gibson and M. Macluskey. Human Papillomaviruses: Prevalence and Association with Survival in Oral Cancer.British Society for Dental Research Conference, 1-4 September 2009, University of Glasgow Caledonian, Scotland UK.

2. A.T. Sailan, M.M. Florence, K. Purdie, M.N.C. de Koning, W.G.V. Quint, P. McLoughlin, P.A. Mossey, J. Gibson and M. Macluskey. Is $p16^{INK4a}$ a surrogate marker for HPVs in oral cancer? International Association for Dental Research Conference, 13-17 July 2010, Barcelona, Spain.

3. A.T. Sailan, M.M. Florence, P. McLoughlin, P.A. Mossey, J. Gibson and M. Macluskey. HPV and p16 in head and neck cancer. International Association for Dental Research Conference, 16-19 March 2011, San Diego, California, USA.