MMP13 AND ISG15 ARE POTENTIAL DRIVER GENES IN ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is an exceptionally aggressive disease with poor prognosis. A major drawback in diagnosis and treatment of OSCC is the lack of knowledge relating to how genetic instability in oral cancer genomes affects oral tumorigenesis. **Objectives:** This current study aimed to determine the copy number alterations (CNAs) in OSCC using array comparative genomic hybridization (array CGH) and to determine the expression of candidate genes. Putative candidate gene was identified and further elucidated to explore its potential role(s) in oral tumorigenesis. **Materials and Methods:** Genome-wide profiling was performed on 75 OSCCs using array CGH. The copy number alterations (CNAs) associated genes that mapped to the amplified and deleted regions were subjected to pathway and network functional analysis using the Ingenuity Pathway Analysis software. The selected putative amplified genes involved in oncogenic networks were further subjected to gene expression analysis using qPCR. The protein expression of the selected putative amplified genes was determined using immunohistochemistry (IHC) technique in the non-cancer oral mucosa, oral epithelial dysplasia (OED) and OSCC samples. Knockdown of the putative amplified gene was performed using small interfering RNA (siRNA) technology in OSCC cell lines and the roles of the gene in cell proliferation, apoptosis, migration and invasion were evaluated. **Results:** In this study, the frequent CNAs were observed on multiple genomic regions, including amplifications on chromosome 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q, and deletions on 3p and 8p. Apart from that, this study also demonstrated the significant association between amplification of chromosome 8q, 11q, 7p and 9p and deletion of 8p with clinico-pathological parameters such as tumour size, lymph node metastasis (LNM) and tumour staging. This study also identified novel candidate genes namely matrix metallopeptidase 13 (MMP13) and

interferon stimulated gene 15 (ISG15) that linked between cell death and survival, cellular movement and cellular development oncogenic network. Furthermore, this study also demonstrated over-expression of MMP13 (chromosome 11q22.2) and ISG15 (chromosome 1p36.33) as prognostic markers in OSCC. Silencing of ISG15 in OSCC cell lines decreased the tumour cell proliferation, migration, invasion, induced apoptosis and increased the cisplatin sensitivity in oral tumourigenesis. **Conclusion:** This current study has identified multiple CNAs including amplifications on chromosome 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q and deletions on 3p and 8p. This study also showed that amplification of the chromosome 7p, 8q, 9p, 11q and genetic signature (+7p8q9p11q) as well as deletion of chromosome 8p was associated with clinico-pathological parameters and poor survival. Apart from that, through the network analysis, the putative amplified genes namely ISG15 and MMP13 were found to be associated with the top oncogenic network namely cell death and survival, cellular movement, cellular development network signalling. This study also has demonstrated the over-expression of MMP13 and ISG15 were associated with lymph node metastasis, tumour staging and poor prognosis. Through the siRNA knockdown of the ISG15 expression inhibited the tumour cell proliferation, migration, invasion and induced cell death in oral tumorigenesis.

ABSTRAK

Latar Belakang: Kanser mulut jenis karsinoma sel skuamus (OSCC) merupakan penyakit yang sangat agresif dengan prognosis yang buruk. Kesulitan utama dalam diagnosis dan rawatan OSCC adalah kekurangan pengetahuan berkaitan dengan ketidakstabilan genetik dalam genom kanser mulut yang mempengaruhi perkembangan kanser. **Objektif:** Tujuan kajian ini adalah untuk menentukan perubahan bilangan salinan (CNAs) dalam OSCC dengan mengunakan tatasusunan penghibridan tatasusunan genomik perbandingan (*array* CGH) dan untuk menyiasatkan pengekspresan calon-calon gen dalam perkembangan kanser mulut. **Bahan dan kaedah:** Pemprofilan genom dilakukan ke atas 75 sampel OSCC mengunakan *array* CGH. Gen-gen yang terpeta dalam linkungan amplifikasi dan deletasi kemudiannya dianalisis dengan menggunakan perisian *Ingenuity Pathway Analysis*. Bagi gen-gen yang mengalami amplifikasi dan terlibat dalam rangkaian onkogenik kemudiannya dinilai semula dengan menggunakan analisis pengeksperesi secara qPCR. Pengekspresan protein bagi gen-gen tersebut kemudian dinilai dengan menggunakan teknik imunohistokimia ke atas sampel-sampel mukosa yang jenis bukan kanser mulut, displasia epitelium oral (OED) dan OSCC. Penyekatan (*knockdown*) gen yang dipilih dijalankan dengan menggunakan teknologi siRNA ke atas titisan sel OSCC dan peranan gen tersebut dalam proliferasi, apoptosis, migrasi dan invasi dinilaikan. **Hasil kajian**: Dalam kajian ini, CNA yang kerap didapati dalam linkungan genomik berbilang merangkumi amplifikasi pada kromosom 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q, dan deletasi pada kromosom 3p dan 8p. Di samping itu, kajian ini juga menunjukkan perhubungan signifikan di antara amplifikasi kromosom pada 8q, 11q, 7p dan 9p dan deletasi pada 8p dengan parameter kliniko-patologi seperti saiz tumor, metastasis nodus limfa dan peringkat tumor. Kajian ini juga menemui calon gen novel iaitu *interferon stimulated*

gene 15 (ISG15) dan matriks metallopeptidase 13 (MMP13) yang mempunyai hubung kait dengan kematian, kelangsungan sel, pergerakan sel dan perkembangan selular dalam proses onkogenik. Selain daripada itu, kajian ini juga menunjukkan pengekspresan berlebihan ISG15 (kromosom 1p36.33) dan MMP13 (kromosom 11q22.2), membolehkan kedua-dua gen ini dijadikan sebagai penanda prognostik untuk OSCC. Penyekatan (*silencing*) ISG15 dalam titisan sel OSCC telah mengurangkan proliferasi, migrasi, invasi sel kanser, malah ia mengaruhkan apoptosis dan meningkatkan kepekaan sel-sel OSCC terhadap rawatan *cisplatin* dalam tumorigenesis. **Kesimpulan**: Kajian ini telah mengenal pasti pelbagai CNA termasuk amplifikasi pada kromosom 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q dan deletasi pada 3p dan 8p. Kajian ini juga menunjukkan amplifikasi pada kromosom 7p, 8q, 9p, 11q dan pengenalan genetik (+7p8q9p11q) serta deletasi pada kromosom 8p berhubung kait dengan parameter kliniko-patologi dan prognosis yang buruk. Selain itu, kajian ini juga telah mengenal pasti pelbagai laluan biologi, fungsi dan rangkaian biologi yang memainkan peranan penting dalam tumorigenesis. Melalui analisis rangkaian, gen ISG15 dan MMP13 yang teramplifikasi mempunyai hubungan dengan puncak rangkaian onkogenik iaitu kematian dan kelangsungan sel, pergerakan sel, perkembangan sel dalam rangkaian signal. Hasil kajian ini juga menunjukkan pengekspresan berlebihan MMP13 dan ISG15 berhubungkait dengan metastasis nodus limfa, peingkat tumor dan prognosis yang buruk. Melalui penyekatan siRNA pada pengekspresan ISG15, ianya telah menghalang proses proliferasi, migrasi, invasi sel tumour, dan malah ia mengaruhkan kematian sel dalam perkembangan kanser mulut.

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LIST OF SYMBOLS AND ABBREVIATIONS

∞	:	Infinity
%	:	Percentage
<	:	Less than
=	:	equal
>	:	More than
⩽	:	Less than or equal
⩾	:	More than or equal
2-ΔΔCT	:	2 delta delta cycle threshold
ANOI	:	Anoctamin 1
ASR	:	age standardized rate
AUC	:	area under curve
AURKA	:	aurora kinase A
BCL-2	:	B-cell lymphoma 2
BIRC2	:	baculoviral IAP repeat containing 2
BRCA	:	Breast Cancer 1, Early Onset
CA9	:	carbonic anhydrase IX
CCND1	:	Cyclin D1
CCNL1	:	Cyclin L1
CDK	:	cyclin-dependent kinase
CDKN2A	:	cyclin-dependent kinase inhibitor 2A
cDNA	:	Complementary Deoxyribonucleic Acid
CGH	:	comparative genomic hybridization
CHL1	:	Cell Adhesion Molecule L1
CIN	:	Chromosomal instability

CLPTM1L	:	Cleft lip and palate transmembrane protein 1
cm	:	centimeter
CNA	:	Copy number alteration
COX-2	:	cyclooxygenase-2
CSMD1	:	CUB And Sushi Multiple Domains 1
CT	:	computerized tomography
CTHRC1	:	Collagen triple helix repeat containing 1
CTTN	:	cortactin
DCTN3	:	dynactin subunit 3
DMEM	:	Dulbecco's Modified Eagle Medium
DNA	:	Deoxyribonucleic Acid
ECM	:	Extracellular matrix
EGFR	:	Epidermal growth factor receptor
EMT	:	epithelial mesenchyme transition
FADD	:	Fas-Associated protein with Death Domain
FBS	:	Fetal bovine serum
FFPE	:	formalin fixed paraffin embedded
FGF	:	Fibroblast growth factors
FHIT	:	Fragile Histidine Triad
FOM	:	Floor of mouth
FOS	:	FBJ Murine Osteosarcoma Viral Oncogene Homolog
FSCN1	:	Fascin 1
gDNA	:	Genomic Deoxyribonucleic Acid
GST	:	glutathione S-transferases
H&E	:	haematoxylin and eosin
HCl	:	Hydrochloric acid

Hg19/GRCh37	:	Human genome 19/ Genome Reference Consortium 37
HNSCC	:	Head and neck squamous cell carcinoma
HPV	:	Human Papillomaviruses Virus
HRR	:	Hazard risk ratio
h-TERT	:	Human telomerase reverse transcriptase
i.e	:	id est
IARC	:	International Agency for Research on Cancer
ICD	:	International Classification of Diseases
ICGC	:	International Cancer Genome Consortium
IFG	:	invasive front grading
IHC	:	Immunohistochemistry
ISG15	:	Interferon stimulated gene 15
Jak	:	Janus kinase
kDa	:	kilodalton
LNM	:	Lymph node metastasis
LOH	:	loss of heterozygosity
LRP12	:	Low Density Lipoprotein Receptor-Related Protein 12
LY6K	:	Lymphocyte Antigen 6 Complex, Locus K
mA	:	milliampere
MAPK	:	mitogen-activated protein kinase
mm	:	milimeter
mM	:	milimolar
MMP13	:	Matrix metallopeptidase 13
MNCR	:	Malaysia National Cancer Registry
MRI	:	magnetic resonance imaging
mRNA	:	Messenger Ribonucleic acid

mTOR	:	mechanistic target of rapamycin
MTT	:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
MUC13	:	Mucin 13
myc	:	myelocytomatosis
N-	:	Negative node
N+	:	Positive node
NF-κB	:	nuclear factor kappa-light-chain
ng/µl	:	Nanogram per microliter
nM	:	nonomolar
nm	:	nanometer
NNK	:	4-(methylnitrosoamino)-1-(3-pyridyl)-1 butanone
NNN	:	nitroso-nor-nicotine
º	:	Degree
OED	:	oral epithelial dysplasia
OPMD	:	oral potentially malignant disorders
ORAOV1	:	Oral Cancer Overexpressed 1
OSCC	:	Oral squamous cell carcinoma
p	:	Long arm
P53	:	Protein 53
P63	:	Protein 63
PAH	:	polycyclic aromatic hydrocarbons
PBST	:	phosphate-buffered saline and Tween 20
PI3K	:	phosphatidylinositol 3-kinase
PKC	:	protein kinase C
PPARγ	:	peroxisome proliferator-activated receptor gamma

PR	:	progesterone receptor
PTK2	:	protein tyrosine kinase 2
PVDF	:	Polyvinylidene fluoride
q	:	Short arm
qPCR	:	Quantitative polymerase chain reaction.
RAS	:	rat sarcoma viral oncogene homolog
RB1	:	retinoblastoma 1
RELA	:	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
RIN	:	Ribonucleic acid integrity number
RIPA	:	Radioimmunoprecipitation assay
RNAi	:	RNA interference
ROC	:	Receiver operating characteristic
ROS	:	reactive oxygen species
rpm	:	Revolutions per minute
RQ	:	Relative quantification
rRNA	:	Ribosomal Ribonucleic acid
SDS PAGE	:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	:	small interfering RNA
SKIL	:	SKI-like proto-oncogene
ß	:	Beta
STAT	:	signal transducer and activator of transcription
STOML2	:	stomatin like 2
TBST	:	Tris-Buffered Saline and Tween 20
TCGA	:	The cancer genome atlas
TGF-ß	:	Transforming growth factor beta
TMA	:	Tissue microarray

TNM	:	Tumour node metastasis classification system
TPM2	:	Tropomyosin 2
TSG	:	Tumour suppressor gene
UICC	:	Union for International Cancer Control
USA	:	United States of America
VCP	:	Valosin Containing Protein
VEGF	:	Vascular endothelial growth factor
volt	:	voltage
WHO	:	World Health Organization
YAP1	:	Yes associated protein 1
µg	:	microgram
µg/ml	:	Microgram per mililiter
µl	:	microliter
µM	:	micromolar

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Oral cancer (including lip) is the 15th most common cancer worldwide, with an estimated 300,000 new cases in 2012 (2.1% of the total cancer cases globally), and the 14th most common cause of death from cancer with an estimated 145,000 deaths (1.8% of the total death due to globally) (Ferlay *et al.*, 2015). More than 90% of oral cancers are oral squamous cell carcinoma (OSCC) and is known to develop at various anatomical subsites which include lip (C00), tongue (C01-02), gum (C03), floor of the mouth (C04), palate (C05) and other and unspecified parts of the mouth (C06) (Neville & Day, 2002). The established risk factors for OSCC are tobacco smoking, alcohol consumption and betel quid chewing (Warnakulasuriya, 2009a). Despite the advances in diagnosis and therapeutic approaches in this lethal disease, the mortality and morbidity rates have not improved over the past decades (Silverman, 2001). The lack of improvement in patient clinical outcomes indicates the lack of knowledge relating to how genetic instability in oral cancer genomes affects oral carcinogenesis (Choi & Myers, 2008; Viet & Schmidt, 2010).

In the year 2007, oral cancer was ranked as the 21st most common cancer in Malaysia (National Cancer Registry Report Malaysia Cancer Statistics) (Omar & Tamin, 2011). Although oral cancer is not amongst the top ten cancers in Malaysia, it was ranked the 3rd most common cancer amongst males [age standardized rate (ASR) = 5.6] and females (ASR = 10.0) of Indian origin. Apart from that, a study conducted on oral cancer patients in Malaysia found that, among those patients, more than 70% of them are diagnosed in their advanced stage with poor survival (data from Malaysian

Oral Cancer Database and Tumour Bank System). Moreover, extensive studies have been reported regarding the significant difference between clinical outcomes with different stages of oral cancer and thus revealing the molecular heterogeneity (Mendez *et al.*, 2009). Therefore, additional predictors and biomarkers are being extensively investigated with newer technologies such as microarray with an aim for better patient management and for the provision of other treatment modalities such as radiotherapy and chemotherapy (Ludwig & Weinstein, 2005; Mishra & Verma, 2010).

In the past, the conventional cytogenetic method such as karyotyping is the ideal technique to identify chromosomal aberrations in genetic disorders (Mitelman *et al.*, 1997). However, the completion of the human genome project in 2011 has enabled the development of new molecular cytogenetic technique namely array based comparative genomic hybridization (array CGH) that could detect the aberrations within the entire genome in a single rapid assay (Nagaraj, 2009). The emergence of this high throughput technology helps to overcome the limitation of the resolution associated with the conventional karyotyping method in diagnosis (Lockwood *et al.*, 2006). For example, the Cancer Genome Atlas (TCGA) (TCGA, 2015) research network and other cancer genome consortiums, such as the International Cancer Genome Consortium (ICGC) (ICGC, 2013), have created large amounts of array and sequencing data at multiple levels for a large number of human cancers. This gives a great opportunity to study the differences and commonalities systematically across different cancer types. These 2 consortiums (India Project Team of the ICGC and TCGA) had identified common mutations in TP53, NOTCH1, CASP8, FAT1, CDKN2A and HRAS in the head and neck cancer (ICGC, 2013; TCGA, 2015). These DNA mutations provide comprehensive set of mutational landscape in the head and neck tumorigenesis and to

promote the discovery of cancer targets, drugs and therapeutic strategies, clinical tests and biomarker development in this cancer.

Oral carcinogenesis is a complex process which results from a multistep pathway involving the accumulation of genetic and copy number alterations (Choi & Myers, 2008). The copy number alterations in terms of amplification and deletions could result in activation of proto-oncogenes and inactivation of tumour suppressor genes (TSGs) that would eventually lead to tumour progression (Pinkel & Albertson, 2005). Development of the genomic profiling analysis emerges as an advance tool to discover potential biomarkers with a clinical value that linked with predictive, prognostic and diagnostic approaches (Ludwig & Weinstein, 2005; Dienstmann *et al.*, 2015). The pattern of CNAs as biomarkers give remarkable significance due to their great impact related with diseases and personalized medicine (Ludwig & Weinstein, 2005). Therefore, the identification of the effective biomarkers for prognosis and diagnosis is an early step in the plan for molecular sub-classification that underlies the pathophysiology of the disease. These specific molecular classifications may have the potential to predict the early stages of the diseases and for the decision making in patient's treatment (prognosis) and personalized medicine (targeted gene therapy) (Ludwig & Weinstein, 2005). The advancement of array CGH enables the identification of candidate CNAs associated genes that could be implicated in tumourigenesis (Lockwood *et al.*, 2006). Apart from that, high resolution array CGH such as oligonucleotide platform enables to detect micro-CNAs as compared to lower resolution array CGH (Przybytkowski *et al.*, 2011). To date, the application of array CGH for identifying Deoxyribonucleic acid (DNA) aberrations has been carried out in oral cancer in order to provide insight into the process of oral tumourigenesis (Viet & Schmidt, 2010). However, the genomic profiles of oral cancer are often diverse due to the influence of various risk factors and ethnicity (Ambatipudi *et al.*, 2011).

To date, conventional treatments for OSCC include surgery, radiotherapy and chemotherapy. Surgery combined with chemotherapy (cisplatin) and radiotherapy can improve overall survival especially in advanced oral cancer patients (da Silva *et al.*, 2012). However, this surgical management often led to severe morbidity due to the disfiguring and functional side effects (Furness *et al.*, 2011). Novel therapeutic alternatives to standard therapy need to be established to improve the prognosis for patients with advanced oral cancer. With the evolvement of RNA interference (RNAi) therapy, one of the molecular targeted therapies approach, it can efficiently overcome some of the major adversities thus associated with the treatment methodologies as RNAi directly targets the cancerous cells at molecular scale (Resnier *et al.*, 2013). By understanding the mechanisms by which RNAi regulates gene expression, this powerful tool can be exploited as an adjunct to the multimodality therapy of various cancers including oral cancer. Research on small interfering RNA (siRNA) demonstrated that combination of the siRNA-directed gene silencing with cisplatin constitutes a valuable and safe approach for the treatment of solid tumours.

It is widely accepted that CNAs are major drivers of genetic instability in cancer (Negrini *et al.*, 2010). The amplification and deletion of chromosomes in cancer can involve the activation or inactivation of oncogenes and tumour suppressor genes, respectively (Pinkel & Albertson, 2005). Therefore, precise mapping of amplified and deleted regions could enable identification of candidate oncogenes and tumour suppressor genes, respectively, that could be responsible for tumour growth (Albertson, 2006). It has been revealed that gene amplification is one way to identify novel oncogenes as it represents selection for expression of gene(s) that promote growth of the tumor (Snijders *et al.*, 2005). Hence, genes mapping within amplicons are candidate oncogenes. On top of that, genome-wide profiling can also be done via high throughput

array CGH to detect even small amplicons that that are particularly informative for identifying candidate oncogenes or pathways (or both). Furthermore, it has been hypothesized that even rarely occurring amplicons would be informative regarding genes crucial in tumor development as the expression may be altered by either copy number changes or other mechanisms by disrupting the upstream or downstream genes in different mechanisms that control a particular pathway (Snijders *et al.*, 2005). Thus, gene mapping in amplicons narrows down the candidate pathways likely to be deregulated in tumors. Furthermore, this approach makes use of identifying the candidate pathways by selection through the regions amplified in cancer rather than focusing on definitive identification of the driver genes for particular amplicons. (Snijders *et al.*, 2005). Nevertheless, further works are required to investigate the function of the candidate oncogene in promoting tumor formation for confirmation.

Among the genetic alterations, CNAs can be found in almost all human malignancies. Several attempts have been made to identify CNAs by searching for new genes that are causative for oral tumourigenesis. In fact, frequent copy number amplification at chromosomes 5p, 14q, 11q, 7p, 17q, 20q, 8q and 3q and frequent copy number deletions at 3p, 8p, 6p, 18q and 4q have been identified in OSCC using array CGH (Ambatipudi *et al.*, 2011; Uchida *et al.*, 2011; Chen *et al.*, 2015; Vincent-Chong *et al.*, 2016). However, the roles of these CNAs in the pathogenesis of OSCC have yet to be elucidated.

Given these facts, the purpose of the current study was to identify novel OSCC related genes by investigating CNAs in the whole genome using array CGH, and by focusing on specific genes included in the CNA region. Furthermore, by performing functional assays of the identified genes, this study aimed to elucidate the role of the

genes in the pathogenesis and progression of OSCC, and to clarify whether the genes have the potential to be new therapeutic targets.

1.2 Aims

The aim of this study was to determine CNAs in OSCC using array CGH and to determine the expression of candidate genes. Putative candidate gene was identified and further elucidated to explore its potential role(s) in oral tumourigenesis.

1.3 Specific objectives

The specific objectives of this study are:

1. To determine and validate the copy number alterations (CNAs) of OSCC using array based comparative genomic hybridization and qPCR copy number assay.
2. To identify the pathways and networks implicated in OSCC pathogenesis.
3. To identify and determine the mRNA and protein expression of candidate genes and their correlation with clinical and socio-demographic parameters as well as survival of patients.
4. To investigate the functional roles of a putative cancer gene using RNAi technique in OSCC cell lines model

CHAPTER 2: LITERATURE REVIEW

2.1 Oral cancer

2.1.1 Definition of oral cancer

World Health Organization (WHO) has documented cancer as a group of disease in which the cells grow uncontrollably and are able to invade to other parts of the body and eventually leading to metastasis (http://www.who.int/topics/cancer/en/). According to National Cancer Institute, any cancer occurring within the oral cavity is known as oral cancer (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0002030/). The World Health Organizations (WHO) International Classification of Disease 10th Revision (ICD-10) Version 2007 has classified the sub-site of oral cancer based on the coding system which comprises of the lip (C00), other and unspecified parts of tongue (C02), gum (C03), floor of mouth (C04), palate (C05) and other and unspecified parts of the mouth (C06) (Johnson 2003).

2.2 Epidemiology of oral cancer

2.2.1 Incidence, mortality and survival

Oral cancer (including lip) is ranked as the 15th most common malignancy in the world (Ferlay *et al.*, 2015). There were 300,000 new cases of oral cancer and 145,000 deaths worldwide in 2012 according to Global Burden of Cancer (GLOBACAN) (Ferlay *et al.*, 2015). In United States of America, oral cancers accounted for 49,200 cases and 12,800 deaths, representing almost 0.3% of all cancer (Ferlay *et al.*, 2015), whereas 61,400 new cases of oral cancers were reported in European Union countries (Ferlay *et al.*, 2015). Developing countries account for almost two-thirds of total oral cancer cases and account for an average of 112,000 deaths (Ferlay *et al.*, 2015). Areas characterized by high incident cases for oral cancer are found in Asia, especially South-Central Asia (Sri Lanka, India, Pakistan), followed by Eastern Asia, specifically Taiwan. Incidence is also high in parts of Europe especially in the Central and Eastern Europe (Hungary, Slovakia and Slovenia) and Western Europe (France). Within The Americas, approximately 18,9000 cases of oral cancer were reported in Northern of America, whereas 10,100 cases were reported from South America (Warnakulasuriya, 2014b; Ferlay *et al.*, 2015). In the United States, five-year oral cancer survival have been improved from 2011 (11%) to 2006 (65%) (Howlader *et al.*, 2010). This is followed by Europe where the five-year oral cancer survival is approximately 50 % (Sant *et al.*, 2009). In other countries such as India, China, the Republic of Korea, Pakistan, Singapore, and Thailand, the five-year oral cancer survival is ranges between 32 and 54 % (Sankaranarayanan *et al.*, 2011; Swaminathan *et al.*, 2011). In Malaysia, the five-year oral cancer survival is lesser than 50% (data from Malaysian Oral Cancer Database and Tumour Bank System).

The Malaysian National Cancer Registry (MNCR) has categorized oral cavity cancers into three distinct groups; namely mouth, tongue and lip cancers (Omar & Tamin, 2011). The combination of these three groups increase the incidence of oral cancer which is 353 cases which comprising lip (n = 9), tongue (n = 93) and mouth (n = 69) (Omar & Tamin, 2011).

2.2.2 Gender, ethnic and age distribution

Oral cancer is more common in men than women with a ratio of 2:1 worldwide (Ferlay *et al.*, 2015). Similarly, in South and Southeast Asian countries such as India, Sri Lanka, Pakistan and Taiwan, the incidence of oral cancer is higher in men compared to women (Warnakulasuriya, 2014b; Ferlay *et al.*, 2015). In Malaysia, the MNCR reported that for year 2007, oral cancer incident cases comprise of 171 males and 182 females with a ratio 1:1 (Omar & Tamin, 2011).

In the United States of America (USA), the Surveillance Epidemiology and End Results (SEER) for oral and pharynx cancer (2009-2013) reported that the age-adjusted incidence rate for blacks and white males were 14.4 cases/million and 17.4 cases/million respectively whereas for blacks and white females were 5.2 cases/million and 6.4 cases/million respectively (www.seer.cancer.gov). Sharp incidence and mortality rate increment were also reported among the non-whites in Germany, Denmark, Scotland, Central and Eastern Europe, Japan, Australia, New Zealand and USA (Kleihues & Stewart, 2003). Interestingly, in Malaysia a marked variation in the incidence of oral cancer was observed among the different ethnic groups that make up the Malaysian population. Although oral cancer was not ranked as the top ten most common cancer overall, it was ranked as the 3rd most

common cancer for both Indian with females having a higher ASR (10.0) compared to males (ASR = 5.6). The Chinese females (ASR = 0.8) have a lower rate than Chinese males (ASR = 1.9), whereas the distribution of cases is almost equal for both sexes among the Malays. These findings confirm the results of a previous study which indicated that the ethnic Indian group has the highest risk for oral cancer amongst the Malaysian population (Zain *et al.*, 1997).

Incidence of oral cancer increases with increasing age throughout the world (Warnakulasuriya, 2009b). Approximately more than 95% of oral cancer cases are reported in people older than 40 years old (Warnakulasuriya, 2009b). In 2009, the database of National Cancer Institute's Surveillance, Epidemiology and the End Results (SEER) reported that the median age of being diagnosed with oral cancer was 62 in USA (Horner *et al.*, 2009). However, there is a gradual increasing trend of incidence and mortality among young adults in USA and European countries (Macfarlane *et al.*, 1994; Shiboski *et al.*, 2005). According to a study by the British Dental Association (2000), approximately 6% of oral cancers were diagnosed in younger patients aged less than 45 years old. In the Indian subcontinent which has one of the highest oral cancer prevalence in the world, oral cancer usually occurs prior to the age of 35 which is mainly due to the practice of tobacco chewing (Johnson, 1991). In Malaysia, a gradually increase of oral cancer prevalence were observed with age more than 40 years old for both male and female (Omar & Tamin, 2011).

2.3 Clinical and histopathological spectrum of oral potentially malignant disorders (OPMDs)

2.3.1 Clinical appearance of OPMDs and oral cancer

Some oral squamous cell carcinomas (OSCC) may be preceded by oral potentially malignant disorders (OPMDs). Although not all OPMDs transform into OSCC, they all belong to a family of disorders characterized by certain genetic and morphological alterations amongst which some have an increased potential for malignant transformation. The presence of an OPMD is an indicator of a future risk of epithelial malignancies (Warnakulasuriya *et al.*, 2007). Leukoplakia, erythroplakia, oral lichen planus and oral submucous fibrosis, are the more common OPMDs. Leukoplakia is a term used to recognize white plaques of questionable risk having excluded other known diseases or disorders that carry no increased risk of cancer (Warnakulasuriya *et al.*, 2007). The incidence and prevalence of leukoplakia varies according to geographic region and study criteria. In 2001, Banoczy et al. (2001) reported that the prevalence of oral leukoplakia varies between 1.1% and 11.7% while the malignant transformation ranges between 2% and 3% according to various studies. According to SEER-Medicare subcohort reported that approximately 0.3% of the US population had a leukoplakia diagnosis (Yanik *et al.*, 2015). In 2003, the estimated reported prevalence of leukoplakia worldwide is approximately 2% (Petti, 2003). The risk of malignant transformation in an oral leukoplakia is dependent on certain factors such as presence of epithelial dysplasia, clinical type and location of the lesion (Petti, 2003). Erythroplakia is defined as a fiery red patch that cannot be categorized clinically or pathologically as any other definable disease. Majority of the erythroplakias undergo malignant transformation but there is a dearth of documented series to calculate a reliable malignant transformation rate (van der Waal, 2009). Histopathologic assessment

for the presence of oral epithelial dysplasia (OED) is considered the current gold standard for predicting malignant transformation of OPMDs. In OED, cells of the normal oral epithelium are replaced by cells showing immature or inappropriate differentiation with a resemblance to cells usually seen in malignancy. Dysplastic features of a stratified squamous epithelium are characterized by cellular atypia and loss of normal maturation and stratification. There is support for the view that in an individual lesion, the more severe the dysplasia the greater the likelihood is of progression to malignancy. However, non-dysplastic lesions may also transform. The latest WHO classification has recommended an objective grading of OED that takes into account the levels of the involved epithelium (Speight, 2007).

OSCC in its early stages may manifest as a white patch (leukoplakia), red patch (erythroplakia) or a mixed red and white lesion (erythroleukoplakia) (Bagan *et al.*, 2010). With progression surface mucosal ulceration or an exophytic mass may develop (Scully & Bagan, 2009). Ulcerated lesions are characterized by raised and rolled margins while exophytic masses may have a fungating or papillary surface (Bagan *et al.*, 2010). With advanced stage disease overt symptoms such as pain, loosening of teeth, dysphagia, trismus, parasthesia and neck masses usually manifest (Bagan *et al.*, 2010).

2.3.2 Histological appearance of OED and OSCC

Traditionally OED has been graded into mild, moderate and severe dysplasia by taking into account a combination of microscopic features that include cytological and architectural changes of the involved epithelium. In grading dysplasia, the thickness (height) to which the cellular and tissue changes extend is also taken into account. In mild forms of dysplasia, recognizable changes are limited to the parabasal layers (lower third); in moderate dysplasia they extend to middle third; and in severe dysplasia, the changes extend to the upper layers (Warnakulasuriya *et al.*, 2008). Accurate histological diagnosis and grading of oral epithelial dysplasia also presents an enormous challenge to the histopathologist. Transformation rates by grade of dysplasia are difficult to establish. One major limitation of using existing histological criteria for dysplasia to predict neoplastic transformation potential is the inherent subjectivity of the grading system itself. Multiple studies have demonstrated low-to-moderate interexaminer consensus for dysplasia grade among experienced oral pathologists. At a workshop coordinated by the World Health Organization (WHO) Collaborating Centre for Oral Cancer and Precancer in the UK on issues related to OPMDs, a two-class classification (no/questionable/mild–low risk; moderate/severe–high risk) system for grading OED was proposed. This was suggested taking into account that reducing the inherent subjectivity in grading dysplasia may increase the likelihood of agreement between pathologists. Kujan *et al.* (2007) tested the new binary system of grading oral dysplasia, and supported this view. However, the biological significance of this system needs to be investigated in longitudinal studies to explore its value in the prediction of malignant transformation risk of OPMDs.

More than 90% of oral cancers are squamous in origin thus termed “oral squamous cell carcinoma”, where it is derived from the surface epithelium. Since the basement membranes are being penetrated, the carcinoma will invade the underlying connective tissues (Eversole, 2001; Nagpal & Das, 2003).

2.4 Etiological factors

The DNA mutation of oral keratinocytes from the consistent exposure of oral mucosa to mutagen agents such as chemical, physical, and microbial elements often contributes to oral cancer (Scully & Bagan, 2009). The use of tobacco products, excessive alcohol consumption, and chewing betel quid are among the etiological factors that have been shown to cause oral cancers by themselves or synergistically (Warnakulasuriya, 2009a).

2.4.1 Smokeless and smoking tobacco

Consumed in combination with betel quid or their substitutes and oral snuff (or guktha, nass, naswar, khaini, mawa, mishri and gudakhu), cigarette smoking is one of the independent risk factor that increases chances of contracting oral cancer by 2-15 times (IARC, 2004b; Rodu & Jansson, 2004; IARC, 2007). Globally, it has been shown that oral cancer risks for smokers are ten times higher than for non-smokers (Warnakulasuriya *et al.*, 2005) while a case-control study showed that the odds for heavy smokers getting oral cancers stood at a ratio of 20.7 (Rodriguez *et al.*, 2004). Other studies document that tobacco smoking heightens that risk by between two to ten fold in men and women respectively (IARC, 2004b).

Among the reasons why tobacco smoking may lead to oral cancer is the presence of more than 300 different carcinogens in tobacco smoke, the major contributory ones being polycyclic aromatic hydrocarbons (PAH), benzo-α-pyrene, tobacco specific nitrosamines including nitroso-nor-nicotine (NNN) and 4-(methylnitrosoamino)-1-(3-pyridyl)-1 butanone (NNK). These carcinogens act as DNA adducts that stimulate oral mucosa epithelium which harm chromosomes and lead to DNA mutations (IARC, 2004b).

2.4.2 Excessive alcohol consumption

Findings from studies by the International Agency for Research on Cancer, regular and excessive alcohol consumption increases the risk of oral cancer by a factor of between two and six (IARC, 2010). When combined, alcohol intake and tobacco smoking increase the risk of contracting oral cancer by 48 times, and account for more than 75% of oral cancer cases reported in developed countries (IARC, 2004b; Rodriguez *et al.*, 2004). Alcohol has the ability to eliminate the lipid content on the membrane of oral mucosa making it more receptive to the infusion of tobacco carcinogens that act as a DNA adduct thereby facilitating oral carcinogenesis (Ogden & Wight, 1998).

According to a study by Mufti (1992), excessive alcohol consumption contributes to an increased occurrence of chromatid breakage in DNA which suggests that alcohol upsets the repair mechanisms of mutated DNA. It has also been reported that heavy consumption of alcohol increases by between five to nine times the possibility to get tongue SCC (Herity *et al.*, 1981). In fact, alcohol drinkers in Asian populations are more at risk of getting oral cancers due possibly to their inactive aldehyde dehydrogenase enzyme which acts as a

detoxifying agent for acetaldehyde which is a known carcinogenic agent (DNA adducts) for oral mucosa (Petti, 2009).

2.4.3 Betel quid chewing

Besides the above, studies from Asian countries particularly in India and Taiwan revealed the risk factor in getting oral cancers from betel quid chewing which is widely practiced in these regions and is labelled as a type 1 carcinogen according to the IARC (2004a). In Malaysia, betel quid chewing is commonly practised by the Indian community, elderly Malays, and the indigenous people from peninsular and east Malaysia, but is less common amongst the young and urban city (Zain *et al.*, 1997).

How the carcinogenicity of betel quid chewing works is still unclear but there are two possibilities. One is the production of potentially carcinogenic nitrosamine in the betel quid chewer's oral cavity and the other is the generation of reactive oxygen species from the auto-oxidation of polyphenols in the areca nut that is enhanced by the alkaline pH of the slaked lime. Both act as contributory factors in increasing the risk exposure of the oral mucosa to these carcinogens (Nair *et al.*, 1985; IARC, 2004a). Besides that, the carcinogenic reactive oxygen species (ROS) and DNA adduct also lead to genetic instability and initiate tumourigenesis through structural changes in the oral mucosa which allow other betel quid compounds to penetrate it (Nair *et al.*, 1985). According to a meta-analysis by Gupta and Johnson (2014), chewing tobacco had a significant and independent correlation with higher risk of OSCC, increasing the possibility of contracting oral cancer by seven times.

2.4.4 Human Papillomaviruses Virus (HPV)

Zur Hausen (1996) notes that HPV is among the most common virus studied in head and neck tumours. HPV types are classified into mucosal or cutaneous depending on their specific lesion sites, with the most common and high risk types being HPV16 and 18 which encode the main viral oncoproteins of E6 and E7. These oncoproteins will further bind to p53 and pRb, thus neutralising tumour suppressor genes which play a role in turning off cell divisions which cause DNA damage. This condition could lead to genomic instability and accumulation of genetic changes and thus lead to malignant progression (Wilczynski *et al.*, 1998).

Till now, the malignant potential of HPV in OSCC has been suspected but not confirmed. Studies on HPV incidence and oral cancer risk have produced widely disparate results ranging from 0 and 100% which could be attributed to geographic differences in population, types of specimens, and HPV detection methods (Kozomara *et al.*, 2005). An IARC case control study identified DNA of HPV in a small subgroup of oral cancers (Herrero *et al.*, 2003). However, a 2008 case study revealed that no HPV was detected in OSCC for non-smoking and non-drinking patients (Siebers *et al.*, 2008). Also, studies have shown that the expression of E6/E7 in HPV DNA positive OSCC was detected in only 6% to 7% cases (Lingen *et al.*, 2013; Reuschenbach *et al.*, 2013) leading to the possibility that HPV infection is not biologically active in most OSCC patients.

2.4.5 Genetic susceptibility

There is a positive correlation between the genetic risk for head and neck cancer and polymorphisms of drug-metabolizing enzymes (Hahn *et al.*, 2002). These enzymes could affect a person's susceptibility to chemical carcinogenesis such as cytochromes P450 (CYPs) that could metabolize polycyclic aromatic hydrocarbons (PAHs) and glutathione S-transferases (GSTs) that are involved in the detoxification of activated metabolites (Hahn *et al.*, 2002). Nevertheless, the three most studied genes for polymorphism in oral cancers are GSTM1, GSTT1, and CYP1A1.

A study among Indian patients with GSTM1 null (deletion) genotype showed an odd ratio (OR) of 1.3 (95% CI 0.37-4.82) (Sreelekha *et al.*, 2001) while that for Japanese patients showed a significant 2.2 chance of risk increase (95% CI 1.4-3.6) for those with null genotype (Sato *et al.*, 1999). Another study among Thais revealed a 2.6-times higher risk (95% CI 1.04-6.5) (Kietthubthew *et al.*, 2001). The situation among Western populations showed contradictory outcomes where a study among the French by Jourenkova-Mironova *et al.* (1999) noted no association between GSTM1 null genotype and the risk of oropharyngeal cancer (OR 0.9, 95% CI 0.5-1.5).

The study by Sreelekha *et al.* (2001) demonstrated that GSTT1 null also imposed an increased risk of 2.5 (95% CI 0.28-21.71) among Indians although Katoh *et al.* (1999) found no association among the null genotype with oral cancer in the Japanese (OR 0.68, 95% CI 0.38-1.22), a finding that is supported by Kietthubthew *et al.* (2001) in their study among such patients in Thailand.

CYP1A1 polymorphism were found to result in an OR of 5.3 (95% CI 1.03-26.28) among Indians (Sreelekha *et al.*, 2001). This finding is supported in other studies which reported a significant 2.6-fold increase in the risk factor (95% CI 1.2-5.7) among Caucasian oral cancer patients (Park *et al.*, 1997) and a 2.3-fold increase (95% CI 1.1-4.7) among their Japanese counterparts (Sato *et al.*, 1999).

However, a preliminary study on 81 oral cancer patients in Jakarta lacked evidence to support any association between polymorphisms of GSTM1, GSTT1, or CYP1A1 and oral cancer occurrence (Amtha *et al.*, 2009).

2.4.6 Diet and nutrition

In 1997, studies by La Vecchia *et al.* (1997) estimated that approximately 15% of oral cancer was caused by imbalances or dietary deficiencies in the European population. This is further demonstrated in Petridou *et al.* (2002) study that vegetables, fruits, micronutrient, dairy product and olive oil plays an important role in protecting against oral cancer with high consumption of riboflavin, iron and magnesium. Meanwhile, Rowland (1991) suggests that Vitamin A could act as a potential protective source from carcinogenesis and lack of them in the diet could possibly lead to cancer metastasis. This is because Vitamin A has the ability to arrest DNA synthesis and can suppress epidermal growth factor receptor (EGFR) which is highly expressed in oral tumourigenesis. Another animal model and laboratory studies by Garewal (1995) mentioned that antioxidant nutrient such as β carotene and vitamin E could play an important role against oral cancer risk. A recent study done by Helen-Ng *et al.* (2012) has indicated that diet plays an important role in oral cancer

prevention where a significant reduced risk of oral cancer was found for consumption of fruit and vegetables in Malaysia population.

2.4.7 Mouthwash

There is also controversy as to the association between the uses of mouthwash containing alcohol with oral cancer (Warnakulasuriya, 2009a). Studies by Winn *et al.* (1991) show that frequent use of mouthwash having concentrations of alcohol exceeding 25% resulted in a higher risk of getting oral and pharyngeal cancer while that by McCullough and Farah (2008) note that such mouthwashes also contribute to oral carcinogenesis. However, a meta-analysis research by La Vecchia (2009) and Gandini *et al.* (2012) confirms that there is no excess oral cancer risks associated with using mouthwash either containing or not having ethanol.

2.5 Prognostic factors

Prognosis is originally derived from a Greek word with term "gignosko" with the meaning "to know". According to National Cancer Institute, prognosis factor is defined as a situation or condition of a patient that can be used to estimate the chance of recovery from a disease or recurrences (http://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=44246). In years, the histopathological assessment of the surgical resection specimen provide information that is essential to determining the post-operative treatment needs and prognosis for oral squamous cell carcinoma patients (Woolgar, 2006). It has also been recommended the idea of the combination between the histological grading and clinical assessment might provide a more precise measure for predicting the outcome of the malignancy and deciding the best treatment for the patient (Anneroth *et al.*, 1987). The prognosis of the oral cancer patient depend largely with clinico-pathological factors such as anatomic location of the lesion, tumour size, nodal involvement, TNM tumour staging, pattern of invasion and histologic differentiation (Massano *et al.*, 2006; Woolgar, 2006; Warnakulasuriya, 2014b).

2.5.1 TNM staging

The tumour-node-metastasis (TNM) system as developed by International Union Against Cancer has been widely accepted and applied in clinical practice to assess the patient's prognosis (Patel & Shah, 2005). This TNM staging systems also used as an international standards for cancer reporting, prognosis evaluation, formulation of treatment strategy, and comparison of treatment results (Sobin *et al.*, 2011). Clinically, the growth potential of the tumour defined by TNM staging includes T, the extent of primary tumour; N, the presence or absence of regional lymph nodes; and M, the presence or absence of distant metastasis (Patel & Shah, 2005). Details of the clinical TNM staging system of oral cancer are shown in Table 2.1. Union for International Cancer Control (UICC) has proposed TNM staging into two classifications which is clinical and pathological classification (Sobin *et al.*, 2011). Clinical classification which is known as the pre-treatment clinical assessment is designated as cTNM and is acquired before treatment planning like physical examination, imaging, endoscopy, biopsy, surgical exploration, and other relevant examinations (basic for the choice of treatment) (Sobin *et al.*, 2011). As for pathological classification or post-surgical histopathological assessment which is designated pTNM, is based on evidence acquired before treatment and supplemented or modified by additional evidence acquired from surgery and from pathological examination that provides additional data to estimate prognosis of the patient treatment (basic for prognosticator or treatment outcome) (Sobin *et al.*, 2011).

In years, the TNM staging system has been utilized to predict the clinical behaviour and appropriate treatment for OSCC (Lindenblatt Rde *et al.*, 2012). Moreover, TNM staging system has also been facilitated in assessment of patient clinical response to therapy and clinical outcomes (Sobin *et al.*, 2011; Lindenblatt Rde *et al.*, 2012). However, this TNM staging system does not efficiently predict patient clinical outcomes due to it does not take into consideration of the tumoural biology, molecular characteristics, the depth of the lesion and histopathologic grade (Broumand *et al.*, 2006; Oliveira *et al.*, 2008). Apart from that, the TNM tumour staging system has been reported to be inadequate in predicting response to non-surgical therapy (Patel & Shah, 2005). Although the cTNM staging of oral cancer can be different from what is found after excision and histopathological examination (termed pTNM) but this tumour staging system remained as the main prognostic factor in OSCC (Macluskey & Ogden, 2000). A review by Warnakulasuriya (2014a) indicated that the TNM classification of OSCC remained to be a reliable criteria for the planning of treatment and to determine patient's prognosis. It is well documented that early stage of OSCC which has not metastases have better prognosis than the late stage of OSCC (Scully & Bagan, 2009). In Malaysia, there were about 64.6% of oral cancer patients who were diagnosed at a late stage (stage 3 and stage 4) (Omar & Tamin, 2011).

Table 2.1: Details of the clinical TNM staging system of oral cancer. Adapted from (Sobin *et al.*, 2011).

Stage	Tumour size (T)	Nodal status (N)	Metastasis (M)
0	T_{is}	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IV	Any T	Any N	M1

T represents tumour size, where T_{is} = carcinoma in situ;

T1 = < 2 cm in greatest diameter,

T2 = 2-4 cm;

T3 = > 4 cm and

T4 tumour with extension to involve adjacent structures, eg. Bone, extrinsic muscles of tongue.

N0 = no clinically detectable enlarged nodes;

N1 = enlarged ipsilateral nodes present ≤ 3 cm

N2 = ipsilateral single > 3-6 cm or ipsilateral multiple ≤ 6 cm;

N3 = any palpable node > 6 cm

M0 = absent metastasis;

M1 = detected distant (blood-borne) metastasis.

2.5.2 Tumour sizes

Tumour size has been documented as the most reliable clinical prognosticator in OSCC and has been widely used for decades in the UICC TNM staging system (Omar, 2013). The prognosis of the OSCC patient has been reported to vary according to tumour size where sizes of T1/T2 (T1- tumour sizes less or equal to 2 cm, T2- tumour sizes more than 2 cm but less than 4 cm) are commonly referred to as "low-risk tumours" whereas T3/T4 (T3- tumour sizes more than 4 cm, T4- tumour invades adjacent structures) are commonly referred to as "high-risk tumours" (Omar, 2013). This is further supported by the recent study by Sawazaki-Calone *et al.* (2015) to determine the prognostic value of tumour sizes in OSCC where their study also revealed that the Cox proportional hazard model confirmed the T size is an independent marker for reduced disease-free survival with a hazard risk ratio (HRR) of 1.40 (95% CI 1.02-1.92, $p = 0.030$). Furthermore, earlier studies conducted by Arduino *et al.* (2008) had shown the influence of tumour sizes (T1/T2 vs T3/T4) as a prognostic factor in OSCC. This study had assessed 334 patients where the authors found that the T3/T4 tumour size is an independent factor in predicting survival of OSCC patients (Arduino *et al.*, 2008). This implies that primary tumour size (T classification) is a reliable prognosticator which influences the patient's outcome with high T sizes indicating a poorer prognosis than those with lower T classification. However, tumour sizes (diameter) alone may not be the best prognostic factor to examine the extent of cancer invasion, response to treatment, and prognosis. Therefore, tumour thickness has been reported to be an important prognostic factor in head and neck cancers. A study conducted by Yuen *et al.* (2000) demonstrated that tumour thickness with up to 3 mm thickness and up to 9 mm of the oral cancer has poorer prognosis revealed that tumour thickness with longer diameter should be considered in the oral cancer management for appropriate treatment.

2.5.3 Lymph node status

It is well documented that OSCC has a high propensity of cervical micrometastases and metastasizes contralaterally because of the rich lymphatic intercommunications relative to submucosal plexus of oral cavity that freely communicate across the midline that facilitate the spread of cancer cells to any area of the neck consequently (Shah, 1990). The role of lymph nodes is known to filter the harmful substances such as dead bacteria, viruses and other dead tissues from the lymphatic fluid and eliminate them from the body (Elmore, 2006). The spreading of cancer cells *via* the lymphatic circulation system and move into lymph node causing the affected lymph nodes to filter out the cancer cells (Elmore, 2006). However, some cancer cells might survive and causing the enlargement and hardening of lymph node that resulted from the metastasis of cancer from primary tumour (Parisi & Glick, 2005). The presence of the enlargement and hardening of lymph node clinically is associated with poor prognosis in OSCC (Shah, 1990). Therefore, the presence of nodal metastasis is the most important prognostic factors for oral cancer (Woolgar, 2006). A study by Woolgar *et al.* (1995) confirmed that lymph node metastases (LNM) displayed profound effects on survival. Furthermore the accurate removal of positive lymph nodes is an important factor in treating OSCC patients. Therefore, the ability to predict LN metastasis at the first biopsy of the primary tumour will significantly improve the management of patients with OSCC (van Hooff *et al.*, 2012). It is highly plausible that LNM helps in the prediction of prognosis and also facilitates in a more effective and appropriate treatment by clinicians. However limited sensitivity of postoperative histology examinations lead to false-positive and false-negative results in patients leading to adverse side effects related to overtreatment and severe risk of fatalities due to overlooked metastases (van Hooff *et al.*, 2012). In a study by Tankere *et al.* (2000), they showed that

the overall survival rate of OSCC patients were 42.8% and 17.5% in the negative node (N-) and positive node (N+) groups, respectively. Contralateral neck metastasis may be associated with higher distant metastasis as spread of tumour across the midline confirms aggressive behaviour.

2.5.4 Primary tumour site

It is widely accepted that the anatomical site of the oral cancer has a prognostic influence in clinical outcomes of the OSCC patients. The plausible factors that influence the prognosis of the sub-site of the oral cancer lesion are the association between the tumour sub-site with the involvement of nodal metastasis, tumour staging, histological grade and pattern of invasion (Woolgar, 2006). The incidence of the involvement of nodal metastases is histologically diagnosed differently among the tumour subsite. For example, the OSCC that form in tongue and retromolar area was highly seen compared to buccal mucosa and gingival/alveolar SCC. The prognosis is also poorer seen in patient with lateral tongue SCC, floor of mouth (FOM) and buccal tumours compared to retromolar SCC patients (Woolgar *et al.*, 1999). This is further supported by other studies also have demonstrated that tongue and FOM OSCC patient have high propensity towards lymph node metastasis due to these regions are known to have a rich and bilateral lymphatic drainage pattern exhibit a higher risk of contralateral metastases compared to other subsite tumours such as lip, retromolar, palate and buccal mucosa SCC (Kowalski *et al.*, 1993; Kowalski *et al.*, 1999). Study conducted by Zelefsky *et al.* (1990) has demonstrated that the treatment results from postoperative radiotherapy of different anatomical sites of advanced OSCC were significant differences in terms of response to combined surgery and radiotherapy. This implies the importance of taking the anatomical site of the oral cancer

into the account for the decision making of appropriate treatment for the different subsite in OSCC.

2.5.5 Histological grading

The histological grading system that initially developed by Broders in 1920 have been proposed by WHO to assess the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity (Pindborg, 1997). The biological activity of OSCC is evaluated and descriptively categorized into well, moderately and poorly differentiated. The modified Broder's classification of well differentiated OSCC has the histological and cytological features closely resemble those of squamous epithelial lining of the oral mucosa. There are varying proportions of basal and squamous cells with intercellular bridges; keratinization is prominent feature; mitotic figures are seen and atypical mitoses or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal. For the moderately differentiated OSCC is described as less keratinization and more nuclear and cellular pleomorphism and there are more mitotic figures and some are subnormal in form with intercellular bridges being less conspicuous. The poorly differentiated OSCC is described as the histological and cytological features showed only slight resemblance to the normal stratified squamous epithelium of the oral mucosa. Keratinization is rarely present and intercellular bridges are extremely scarce, there is frequent mitotic activity and atypical mitoses can readily be found with obvious cellular and nuclear pleomorphism and multinucleated cells may be present (Pindborg, 1997). There is consistent evidence of the value of tumour histological grade in determining prognosis and have shown that poorly differentiate OSCC was associated to poor prognosis in OSCC patient (Arthur & Farr, 1972; Langdon *et al.*, 1977). However, there were other

studies have showed that Broders' histological grading is no longer have prognostic impact in OSCC (Pindborg, 1997; O-Charoenrat *et al.*, 2003). The plausible reasons are due the histological heterogeneity and inadequate sampling, reliance on structural characteristics of the tumour cells and evaluation of tumour cells in isolation from the supporting stroma and host tissues.

2.5.6 Pattern of invasion

Clinical assessment by the TNM tumour staging system is widely and routinely used to determine the prognosis and treatment options for patients with OSCC (Sawair *et al.*, 2003). However, the major drawback of this tumour staging system is that it overlooks the histological characteristics of tumours (Anneroth *et al.*, 1987). Therefore, in 1989, Bryne has developed a malignancy grading system focusing only on the invasive front of the tumour. Typically, this invasive front grading (IFG) system are graded and assigned points from 1 to 4, the score for each variable is summed to provide a total malignancy score for each tumour. The observation of tumour cell at the most invasive part of the tumour believe to have a more aggressive behaviour compared to the central or superficial tumour cell suggesting that the invasive front of the tumour cell have the ability to invade to the surrounding tissue structures, including vessels, and lead to metastasise (Bankfalvi & Piffko, 2000). Several studies have been demonstrated the prognostic impact of this invasive front as a prognostic factor that may influence the treatment of OSCC (Sudbo *et al.*, 2000; Chang *et al.*, 2010; Almangush *et al.*, 2014).

2.6 Oral cancer management

Oral cancer is diagnosed by physical examination of the oral cavity and neck as well as computerized tomography (CT) and magnetic resonance imaging (MRI) imaging to evaluate the participation of adjacent structures, such as bones and soft tissues (Sankaranarayanan *et al.*, 2015). Although surgery and radiotherapy are the common treatment given for oral cancer patients, these modalities can lead to severe morbidity due to disfiguring and functional side effects (Furness *et al.*, 2011).

2.6.1 Treatment of early stage oral cancer

Diagnosis of early oral cancer lesions are often followed by surgery and/or radiotherapy (Sankaranarayanan *et al.*, 2015). Treatment are catered based on the tumour location, cosmetic and functional consequences, age of the patient, presence of other diseases, patient's preference, and the availability of expertise (Sankaranarayanan *et al.*, 2015).

Majority of the early stage oral cancers can be locally excised or treated with radiotherapy, often with no or minimal functional and physical damage. For patients with stage I tongue cancer and stage II cancers at other oral sites, who may be at high risk of microscopic involvement of the neck nodes, elective neck dissection to remove lymph nodes may be considered (Woolgar, 2006; El-Naaj *et al.*, 2011; Vijayakumar *et al.*, 2011).

External beam radiotherapy and brachytherapy which is using radioactive sources implanted in the tumour- either alone or in combination, is an alternative to surgery for early stage oral cancers (Sankaranarayanan *et al.*, 2015). These treatment modalities have been reported to have improved the clinical outcome of oral cancers (Fujita *et al.*, 1999; Marsiglia *et al.*, 2002). However, brachytherapy alone, which does not treat regional nodes sufficiently, is not endorsed for deep infiltrative cancers due to their nature to spread to regional lymph nodes. More advanced recent techniques, such as three-dimensional conformal radiotherapy and intensity modulated radiotherapy, can reduce the side effects of conventional radiotherapy by improving the dose and delivery of radiotherapy, but these treatments require much sophisticated equipment and they generally are more expensive (Sankaranarayanan *et al.*, 2015).

2.6.2 Treatment of advanced oral cancer

Locally advanced cancers are aggressive tumours with high locoregional treatment failure rates. Therefore, a combined modality strategy integrating surgery, radiotherapy and/or chemotherapy is often preferred for the management of the disease, although the treatment option is largely influenced by factors such as functional and cosmetic outcomes and the available expertise (Sankaranarayanan *et al.*, 2015). Surgery followed by postoperative radiotherapy is the preferred modality for patients with deep infiltrative tumours and those with bone infiltration (Lundahl *et al.*, 1998). For patients with surgical margins with malignant characteristics indicating incomplete excision of the tumour, postoperative concurrent chemoradiation is often given (Bernier *et al.*, 2004; Cooper *et al.*, 2004). The use of chemotherapy prior to surgery can be beneficial as it may eliminate the need to remove the mandible (Licitra *et al.*, 2003).

Primary radiotherapy, either as single modality or in combination with chemotherapy, is also a sensible option for locally advanced tumours without bone involvement as well as for patients with unresectable tumour, who are medically unfit for surgery, or who are likely to have unfavorable functional and cosmetic outcomes with surgery (Sankaranarayanan *et al.*, 2015). Incorporating chemotherapy with surgery or radiotherapy is beneficial in younger patients with good health condition (Blanchard *et al.*, 2011).

The use of chemoradiotherapy seems to be favorable in unresectable late stage (III/IV) OSCCs (Argiris *et al.*, 2008), although its use in distant metastasis may not be as useful, due to the complications of treatment such as mucositis, dermatitis, and myelosuppression (Argiris *et al.*, 2008). In addition, radiotherapy and chemotherapy, or either as single modality or combined, can confer long-term side effects that may aggravate the quality of life of the patients. Therefore, much precise understanding of OSCC is imperative for future treatments (Sankaranarayanan *et al.*, 2015).

2.6.3 Molecular targeted gene therapy in OSCC

Despite much advancement in the multidisciplinary treatment comprising surgery, chemotherapy, and/or radiation, the overall survival rate of OSCC patients has not improved significantly. Thus, new therapeutic alternatives are crucial to improve the prognosis of patients, especially those with advanced disease (Hamakawa *et al.*, 2008). Targeted therapies might be a good solution for this problem as they are becoming popular in cancer research. This modality uses drugs or other substances that specifically target a molecule involved in the growth, progression, and spread of cancer, thereby reducing tumour growth and metastasis (Hamakawa *et al.*, 2008). In the recent years, many different targeted therapies have been approved for the use of cancer management, including hormone therapies, signal transduction inhibitors, gene expression modulators, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules (Hamakawa *et al.*, 2008). The generally agreed strategy to identify potential targets for this therapy is to compare the amounts of selected proteins, usually with a role in cell growth, survival, motility or angiogenesis, between the non-disease and cancer cells (Hamakawa *et al.*, 2008). Proteins that are abundantly expressed in cancer cells compared to normal cells would be good candidate targets. Alternatively, mutant proteins with functional significance, which can drive tumourigenesis, can also be potential targets for therapy. In addition, identifying cancer-specific abnormalities in chromosomes can also lead to the development of targeted therapy (http://www.cancer.gov/about-cancer/treatment/types/targeted-therapies/targeted-therapies-fact-sheet).

Over the years, many molecular targeted therapies have been proposed and shown to have promising anticancer effects. Those targets which are much investigated in OSCC,

includes the epidermal growth factor receptor (EGFR), cyclooxygenase-2 (COX-2), peroxisome proliferator-activated receptor γ (PPARγ), and the progesterone receptor (PR) (Hamakawa *et al.*, 2008). Gefitinib ("Iressa," ZD1839) is a small molecule EGFR tyrosine kinase inhibitor which can inhibit the proliferation of OSCC cell lines in both *in vitro* and *in vivo* models. The agent also showed improved outcome when given after radiotherapy (Hamakawa *et al.*, 2008). This was due to the effect of the gefitinib/ radiotherapy combination on the downstream survival regulator, p38 mitogen-activated protein kinase (MAPK) and MAP kinase (MEK) 1/2 autophosphorylation (Hamakawa *et al.*, 2008). Treatment with celecoxib, a COX-2 selective inhibitor, enhanced the radiosensitivity of HSC-2 cells, which constitutively expressed COX-2 (Hamakawa *et al.*, 2008). Apart from EGFR and COX-2, another promising molecular target is the PPARγ, which is a member of the nuclear receptor superfamily of ligand-activated transcription factors. Recent findings have shown that PPARγ ligands induce cellular differentiation and inhibit cell growth in multiple malignancies, thereby hinting the potential use of synthetic PPARγ ligands for molecular targeting of oral cancer (Hamakawa *et al.*, 2008). Another promising targeted gene therapy known as RNA interference (RNAi) based gene therapy. This approach is using either small interfering RNA (siRNA) or plasmid or viral vector mediated delivery of short hairpin RNA to inhibit the expression of the particular oncogenes (MYC, FOS and RAS) which enable to reduce the tumour growth in oral tumourigenesis and thus act as a powerful anti-tumour agent with minimum toxicity (Bertrand *et al.*, 2002; Xi & Grandis, 2003).

2.7 Carcinogenesis

Certain enzymes, upon undergoing genetic instability, accelerate the pathogenic pathways that are involved in carcinogenesis. For examples, DNA replication and repairing, chromosomal instability, apoptosis and cell cycle regulation are often disrupted as a result of DNA damage (Beckman & Loeb, 2005). In oral carcinogenesis, the multistep process involves the accumulation of genetic changes such as chromosomal aberrations, DNA mutations and epigenetic alterations impede the normal cellular processes and lead to abnormal amplification of centrosomes, defects in DNA repair mechanism, uncontrolled cell proliferation and reduction of apoptosis (Scully *et al.*, 2000; Weinstein, 2002; Choi & Myers, 2008). As a consequence of these events, different phenotypic changes occur and result in elevated malignancy and aggressive behaviour of the oral tumour cells.

Cancer can be defined as a disease caused by the unregulated DNA replication, cell growth, cell division and survival which differs the tumour cells from their normal counterparts (Evan & Vousden, 2001). Hence, cancer is the condition where the normal co-operative cell behaviours that facilitate the multicellularity functions are lost (Abbott *et al.*, 2006). In another word, cancer cells become unresponsive to the usual controls on proliferation and follow their own agenda for reproduction instead, which is beyond the constraints of normal cells (Weinberg, 1996).

The three main steps that are needed for the development of cancer are described to be the initiation, promotion and progression (Farber, 1984). Starting with the initiation process when there are irreversible cellular changes or mutations that cause both the irreversible or reversible DNA damages. This step can arise spontaneously or be induced by exposure to a

specific carcinogen. In fact, the initiated cells are still not characterized as tumour cells because they have not acquired the autonomy of growth yet. Besides, the DNA instability may remain undetected throughout life (Okey *et al.*, 1998). During the promotion stage, it is generally characterized with further unchecked proliferation of the mutated cells from the initiation phase. This results in a rapid increase of the tumour size (Farber, 1984) and the process can be enhanced by chronic exposure to carcinogenic stimuli which causes the changes of the initiated cells, leading to neoplastic transformation to favor tumour growth (Okey *et al.*, 1998). Lastly, the final stage of cancer development is the progression stage where successive mutations will give rise to increasingly malignant sub-populations. The pre-neoplastic cells will tend to become more committed to malignant development and this whole process involves accumulation of further gene mutations leading to heterogeneity in cell population (Farber, 1984). Overall, the cells lose their adherence property and detach from the tumour mass to invade the neighbouring tissues or enter the circulating blood and lymph to be transported to other tissues or organs as the tumour progression advances. Once the tumour cells move away from the primary sites, distant metastases occur and they form the secondary tumours (Okey *et al.*, 1998).

2.7.1 Oncogene and tumour suppressor gene

Both the events of gene amplification and deletion lead to tumourigenesis. Gene amplification in tumourigenesis is often associated with oncogene activation (Coleman & Tsongalis, 2002) while the loss of chromosomal contents is linked to the inactivation of the tumour suppressor genes (TSG) (Fröhling & Döhner, 2008). In general, the progression and pathogenesis of OSCC results from a list of genetic events related to the activation and inactivation of oncogenes and TSG respectively (Argiris *et al.*, 2008).

2.7.1.1 Oncogenes

Oncogenes are derived from the gain of functions in cellular proto-oncogenes, which occur through the alteration of a few common mechanisms such as mutation, chromosomal rearrangement (translocations and inversions), and gene amplifications (Croce, 2008). There are a total of six functional groups of oncogenes which are further categorized into different subgroups, which are the transcription factors, growth factors, growth factor receptors, chromatin remodelers, signal transducers and apoptosis regulators (anti-apoptotic regulators) (Croce, 2008). According to Field (1995), amplification of oncogenes is more prominent during tumour progression which is enhanced by certain encoded proteins through checkpoint dys-regulation of the cell cycle. C-myc, int-2, hst-1, CCND1 and EGFR are among the oncogenes associated with oral carcinogenesis (Todd *et al.*, 1997).

Previous studies have indicated the amplification and over-expression of EGFR in oral cancers (Ishitoya *et al.*, 1989; Chen *et al.*, 2003). In normal conditions, EGFR genes are involved in transmembrane tyrosine-specific phosphokinase activity. The binding of EGFR to their ligands leads to phosphorylation of EGFR tyrosine residues to dimerization and activation. Therefore, amplification of this gene would lead to the continuous activation of important kinase pathways. This subsequently favours unceasing cell growth and proliferation, invasion, metastasis and angiogenesis in oral cancer (Yano *et al.*, 2003; Ciardiello *et al.*, 2004; Kalyankrishna & Grandis, 2006).

Moreover, another popular oncogene in oral cancer is the v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) gene (Xia *et al.*, 1997), which is activated

through heterodimerzation with other receptors. Then, the MAPK or phosphatidylinositol 3-kinase (PI3K)-AKT activated pathways are activated, which play a role in enhancing tumour invasion, cell proliferation, differentiation, adhesion and cell migration in cancer (Olayioye *et al.*, 2000).

The association between the amplification of CCND1 with tumour aggressiveness for head and neck carcinoma has also been previously reported (Callender *et al.*, 1994). Generally, the CCND1 is responsible for the cell proliferation promoter in G1 phase of the cell cycle under normal conditions. However, when over-expressed as a result of gene amplification, cancer progression in tumourigenesis will be greatly enhanced (Hunter & Pines, 1991).

2.7.1.2 Tumour suppressor genes

On the other hand, the inactivation or deletion of tumour suppressor genes (TSG) is responsible for initiating cancer progression (Cooper, 2000) as this group of genes are the negative growth regulators. Thus, they regulate cellular trafficking, DNA damage response and apoptosis in a recessive fashion. Generally, TSG is defined as a genetic element whose loss or inactivation allows a cell to display one or the other phenotype of neoplastic growth deregulation (Weinberg, 1991). Therefore, one of the major events during carcinogenesis is inclusive of the loss of function of TSG (Munger, 2002). While it is common to find a few different inactivated TSG in the same tumour, it is also not a surprise to find the same TSG being inactivated in different types of tumours. Loss of these genes basically promotes cell cycle proliferation, signal transduction, angiogenesis and tumour growth (Sager, 1989).

Since TSG are recessive, they require two mutated alleles for tumour formation and it was reported in a two-hit mechanism/theory that stated both the alleles needed to be inactivated in order to promote malignant growth proposed by Knudson's review paper (Knudson, 1971, 2001).

As a matter of fact, TSG has been categorized into 2 groups which were "gatekeepers" or "caretakers" (Kinzler & Vogelstein, 1997) and "landscapers" (Kinzler & Vogelstein, 1998). The "gatekeeper" genes' main functions are to inhibit the tumour growth, suppress neoplasia and promote cell death. Hence, the loss of these genes is rate-limiting in multi-stage tumourigenesis (Kinzler & Vogelstein, 1997). Examples for these genes are the RB1 and TP53. On the contrary, "caretaker" genes work as DNA maintenance genes to suppress growth by ensuring the fidelity of the DNA code through effective repair of DNA damage and maintain genome integrity (Kinzler & Vogelstein, 1997; Russo *et al.*, 2006). Examples for this group of genes include the breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2). Meanwhile, "landscaper" genes actually work through a less direct mechanism by affecting tumour microenvironment in which tumour cells grow such as extracellular matrix (ECM) protein, cell surface markers, adhesion proteins and survival factors. Therefore, malignancies follow upon the loss of "landscaper" genes' functions (Kinzler & Vogelstein, 1998). Consequently, Thiery (2002) reported that the loss of E-cadherin and alpha-catenin in epithelial cell junction and epithelial stromal cell interaction induce epithelial mesenchyme transition (EMT) in carcinoma progression.

The loss of function of FHIT, RB1, TP53 and CDKN2A genes has been previously reported in the head and neck cancer, particularly the SCC cases (Virgilio *et al.*, 1996;

Koontongkaew *et al.*, 2000). Basically, a particular TSG can undergo biallelic inactivation through chromosomal allelic loss. Examples of such event include the loss of heterozygosity (LOH)-allelic deletion, point mutation and deletion of both alleles (Knudson, 2001). Biallelic inactivation is able to unmask recessive mutations which, in a way are able to bring about changes on the cell phenotype (Yokota & Sugimura, 1993).

2.8 Hallmarks of cancer

Hanahan and Weinberg (2000) have identified six hallmarks for distinguishing cancer cells from their normal counterpart, which include the self-sufficiency of growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, immortality or unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis. However, as cancer research advanced significantly, Hanahan and Weinberg (2011) added two new emerging hallmark capabilities, which are the reprogramming of energy metabolism and evading immune destruction into the present list.

2.8.1 Self-sufficiency of growth signals

Under normal conditions, cells require soluble and membrane bound growth factors which function like a signal for the inert cells to be transformed into proliferative cells (Hanahan & Weinberg, 2000). Basically, these growth signals are like the ligands of receptors that are transduced from the cell surface to activate specific intracellular signaling pathways which increases cell proliferation activities (Aaronson, 1991; Hanahan & Weinberg, 2000). The dys-regulation and increased expression of these growth signals form the hyper-responsive tumour cells and subsequently become one of the most important drivers of self-sufficiency growth in oral cancer (Todd *et al.*, 1991). Examples of

intracellular growth signalling transducing proteins include EGFR, CCND1 and RAS. They play the same role as downstream mediators of growth factor signaling in OSCC.

EGFR encodes a membrane-bound receptor tyrosine kinase, in which the autophosphorylation of its receptors will result in a series of intracellular signal cascading events that includes the activation of the RAS/Raf/MAPK, phosphatidylinositol-3-kinase (PI3K), mechanistic target of rapamycin (mTOR), Janus kinase (Jak), signal transducer and activator of transcription (STAT), and protein kinase C (PKC) pathways (Rogers *et al.*, 2005; Kalyankrishna & Grandis, 2006). These pathways are well known to be involved in the cell proliferation, survival, invasion, metastasis and angiogenesis in tumourigenesis (Reuter *et al.*, 2007). Secondly, the Ras encodes protein p21 *via* mutation that leads to tumour cell growth regulation and mitogenic cell signaling transduction from cell surface to nucleus. Meanwhile, the proto-oncogene, CCND1 encodes a positive regulator in cell cycle that initiates DNA synthesis. Therefore, the activation of CCND1 results in uncontrollable cell proliferation in tumourigenesis.

2.8.2 Insensitivity of growth-inhibitory signals

Hunter and Pines (1994) discovered that when the tumour suppressor genes undergo the loss of expression in cell cycle inhibitory protein (which in turn, is controlled by the growth inhibitory signals such as p53 and pRB), it will lead to an increased cell proliferation rate. This particular hallmark may be observed in the dys-regulated interactions between the growth inhibitory signals and cyclin dependent kinase (CDK) in cancer. As a result of this, the cell cycle progression of cancer patients is relatively higher and faster (Serrano *et al.*, 1993).

2.8.3 Evasion of programmed cell death

Additionally, Hanahan and Weinberg (2000) stated that not only does uncontrolled cell proliferation induces tumour growth but also the ability of tumour cells to maintain cell proliferation and prevent senescence *via* apoptosis. Oren (1992) as well as Manning and Patierno (1996) reported that cancer cells have increased survival rate and evade apoptosis, unlike the normal counterparts. This event is achieved in various ways in oral cancer. One form is the over-expression of B-cell lymphoma 2 (Bcl-2) which is an anti-apoptotic regulatory protein in cancer (Pezzella *et al.,* 1993). It has been suggested that Bcl-xL inhibition can be an effective treatment for OSCC patients who develop resistancy towards cisplatin (Oliver *et al.,* 2004).

2.8.4 Immortality or unlimited replicative potential

The chromosomal telomere shortens each time after every cell cycle decreases the life span of the cells (Hanahan & Weinberg, 2000). But cancer cells are immortal with infinite lifespan due to their ability to replicate indefinitely by increasing the length of telomeres (Hayflick, 1997). Cancer patients tend to have upregulated telomerase enzyme which protects the tumour cells against telomerase shortening. The enzymes work by extending the end of telomere *via* reverse transcription (Shay & Wright, 2006) to increase the cancerous cells' life span. In fact, several studies have found that the over-expression of human telomerase catalytic subunit gene (hTERT) was significantly associated with poor prognosis in oral cancer (Kannan *et al.,* 1997; Gordon *et al.,* 2003).

2.8.5 Sustained angiogenesis

The onset of angiogenesis was reported to be associated with tumour growth and metastasis (Folkman, 1990). In a study by Folkman (2006), sufficient blood supply is said to provide nutrients to tumours *via* the angiogenic mechanism which promotes tumour growth and development. Furthermore, Hanahan and Folkman (1996) described the role of angiogenic switch in tumour progression, which is dependent on the balance mechanism between the pro-angiogenic signals and anti-angiogenic signals. The pro-angiogenic state is favoured in tumourigenesis as it produces new blood vessels for cancer tissues. The role of angiogenic switch has been well reported in a study by Udagawa *et al.* (2002) which showed that the increase in the expression of vascular endothelial growth factor (VEFG) through transfection was linked to the expansion of tumour in mice. In fact, it has been shown that the expression of VEGF is higher than normal oral mucosa tissues in an oral cancer study (Denhart *et al.*, 1997). This suggests that tumour angiogenesis is correlated with tumour progression and aggressiveness in the oral mucosa.

2.8.6 Tissue invasion and metastasis

Above all the vital cancer hallmarks, the most important characteristic that differentiates cancer cells from their normal counterpart is their ability to form metastasis and invade into the surrounding tissues. These events associated with poor prognosis of cancer patients (Liotta, 1986; Sporn, 1996; Hanahan & Weinberg, 2000), and contributing to tumour invasion are not fully understood. Nevertheless, it has been reported by Liotta (1986) that cancer cells interact with the surrounding matrix at various stages in its metastatic cascade through a series of complex processes such as involving intravasation through angiogenesis, extravasation and growth into surrounding tissues, survival in the

bloodstream and new organ (secondary organ), cytoskeleton remodeling, initiation and maintenance of growth and neo-angiogenesis in the metastatic tumour (Chambers *et al.*, 2002).

In addition, Thiery (2002) found that metastasis was actually associated with EMT. This finding was also supported by Gumbiner (1996) which reported that loss of E-cadherin expression during EMT led to increased cell motility and invasion. In general, there was a set of transcriptional factors involved in EMT and related migratory processes such as Snail, Slug, Twist, Zeb 1 and Zeb 2. They were often displayed as the important invasion programmers in carcinoma formation (Thiery, 2002).

2.8.7 Reprogramming energy metabolism

Chronic and uncontrolled cell proliferation represents the main element of neoplastic disease which involves not only the dys-regulated control of cell proliferation but also the corresponding adjustments of energy metabolism to support cell growth and development. One of the characteristics of malignant cells include the increased glycolytic metabolism, which is associated with the activation of oncogenes such as RAS, MYC and the deactivation of tumour suppressor genes like TP53 in tumour cells. These genes were selected in identifying the hallmark capabilities of cell proliferation, avoidance of cytostatic controls, and attenuation of apoptosis (DeBerardinis *et al.*, 2008; Jones & Thompson, 2009).

2.8.8 Evading immune destruction

According to Hanahan and Weinberg (2011), they have characterized that tumour cells are capable of escape from the immune defence system or have minimized magnitude of immunological killing. Therefore, the tumour cells are able to avoid eradication and this has been characterized into one of the cancer hallmarks. Among the mechanisms involved in the evasion of immune attack is the selection of tumour variants resistant to immune effectors, and progressive formation of an immune suppressive environment within the tumour. Cancer cells that are vulnerable to immunology attack present tumour antigens that can be recognized and eliminated by the immune system, but constant cell division as a result of genetic instability tends to decrease immunogenicity.

2.9 Genetic Instability

Chromosomal instability (CIN) is among the most common forms of genomic instabilities (Loeb, 2001; Negrini *et al.*, 2010). Hence, being one of the distinctive hallmarks of most cancers, CIN is characterized by changes in chromosomal structure – which includes inversions, point mutations and translocations (Albertson *et al.*, 2003) and numerical aberrations such as amplification and deletions, which consequently are able to cause uncontrolled cell proliferation, altered cell morphology and tumour progression (Lengauer *et al.*, 1998; Gollin, 2005). Basically, balanced (reciprocal) and unbalanced (non-reciprocal) are the two categories of structural aberrations, in which, reciprocal alteration is said to be the exchange of chromosome parts of non-homologous chromosomes and results in no genetic content to be lost or gained, whereas, in non-reciprocal alteration, the exchange is unequal, causing extra or missing copies of genes and chromosomal regions (Albertson *et al.*, 2003).

2.9.1 Chromosomal instability

Chromosomal instability (CIN) occurs in cells that have increased amount of unstable chromosome content, which includes features like DNA translocation, aneuploidy (loss/gain of whole or portions of chromosome) changes in gene copy number (amplification and deletion) and chromosomal rearrangement (Lobo, 2008), that are aroused from the event of abnormal mitosis (Loeb, 2001).

Oral cancer, just like all human cancer types – undergoes chromosomal and genetic aberrations (Reshmi & Gollin, 2005). It has been reported that CIN contributes to tumour initiation and progression and is the central issue in cancer biology. The normal cellular processes - such as cell signaling, replication, and apoptosis will be negatively affected by chromosomal abbreviations. This subsequently may result in uncontrollable cell proliferations and defects in DNA repair mechanisms which are often the key factors for tumourigenesis (Loeb *et al.*, 2003). Furthermore, Katayama *et al.* (2003) has also reported that over-expression of aurora kinase A (AURKA) gene in cancer may lead to failure in maintaining stable chromosomal contents due to defective centrosome maturation, bipolar spindle assembly and mitotic entry. Moreover, defective functioning of mitotic checkpoint machineries such as centrosomes, microtubules, kinetochores, loss of spindle check point, abnormalities of double strand break repair and telomere dysfunction in mitosis are primarily caused by the failure in separation of sister chromatids, before the attachment of microtubules during cell division (Schvartzman *et al.*, 2010). Such defects are reported to have contributed to changes in chromosomal numbers and structures which generally result in CIN (Reshmi & Gollin, 2005; Schvartzman *et al.*, 2010).

According to Saunders *et al.* (2000) and Gisselsson *et al.* (2002), the involvement of extrinsic cytoskeletal aberrations such as multipolar spindles and alterations in centrosome number were detected in few oral cancer studies. Both events were said to be responsible for CIN. For instance, Saunders *et al.* (2000) had successfully observed various degrees of multipolar spindles from OSCC cell lines (for examples, different level of chromosomal capture and alignment) while Gisselsson *et al.* (2002) had shown the alterations of centrosome number in oral cancer and suggested that it was the internuclear connection that caused the prevention of cytokinesis and lead to the duplication of both chromosome and centrosome number.

2.9.2 Driver and passenger mutation

Cancer is a progressive event that occurs from the process of multiple somatic mutations. The changes in somatic cells, which include base substitutions, deletions, insertions and rearrangements, are usually caused by the frequent exposure of mutagens from both internal and external environments, result in permanent alteration of DNA sequences (Vogelstein *et al.*, 2013). For every somatic mutation that can be found in the human cancer genome, it can be further classified into two different categories – which are the driver gene mutation and passenger gene mutation. They are basically distinguished by their roles in the development of cancer respectively. Generally, driver gene mutations are characterized by their selective growth advantages and contribution in tumourigenesis (Pon & Marra, 2015). Hence, they are also known as the cancer genes. The number of driver gene mutations present in a tumour may also be associated with cancer development and treatment complications. Meanwhile, passenger gene mutations are often biologically inert or have little impact on cell function. In fact, it has been reported that passenger gene

mutations are most likely already present in cells before the acquisition of the driver gene mutations (Stratton *et al.*, 2009).

To this day, the complexity and uncertainties underlying the cancer genome remains as the biggest challenge for researchers to differentiate between the two types of mutations. In addition, there are also papers outlining that driver genes may also contain passenger gene mutation and on the other hand, passenger genes can be converted into driver genes under environmental changes following cancer treatments (Stratton *et al.*, 2009; Vogelstein *et al.*, 2013). By far, large-scale sequencing studies comprising a huge set of samples are the most promising approach to discover and explore driver gene involvement in different types of cancers. Besides, as each individual tumour has its unique set of genetic mutational landscape, a combination of different methods including both genetic and functional analyses are crucial to precisely identify true driver gene mutations (Haber & Settleman, 2007).

One of the approaches to explore the genetic mutational landscape of HNSCC is using the exome sequencing technology. The recent study conducted by Agrawal et al. (2011) had significantly found more mutations occurred in HPV negative or tobacco-associated OSCC compared to HPV positive related oropharyngeal cancer (Agrawal *et al.*, 2011). Taken together with Stransky et al. (2011) study, these two genetic mutational landscape studies revealed a two- to five-fold increase in mutation rates in the mutated genes namely TP53, CDKN2A, PIK3CA, HRAS and NOTCH1 in HNSCC with HPV negative compared to HPV positive related HNSCC suggesting that the subtypes have distinct genetic mutational landscape. In 2013, an integrative genomic analysis of OSCC conducted by

Pickering et al. (2013) had identified a frequent mutation of CASP8 with few CNAs suggesting this inverse correlation may give an understanding to the mechanisms that induce the genomic instability in oral tumorigenesis.

2.10 Model of OSCC

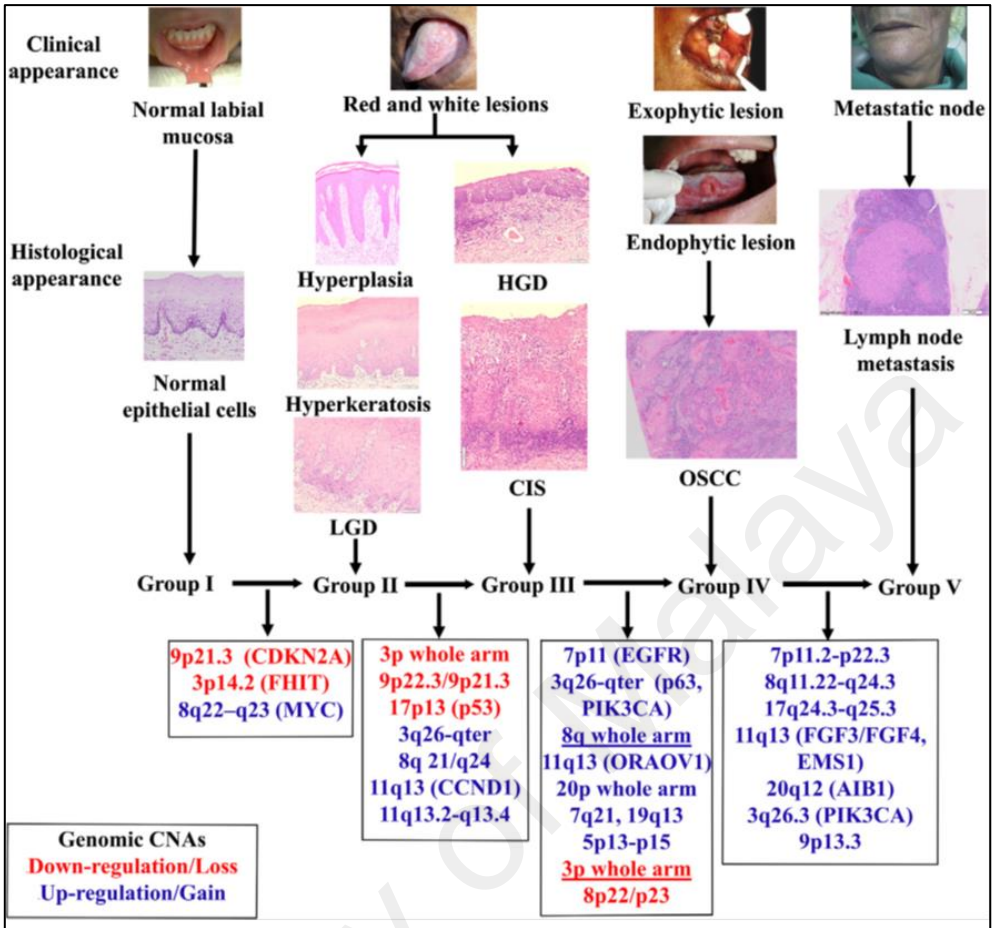
A recent study that conducted in Oral Cancer Research and Coordinating Center (OCRCC), Faculty of Dentistry in University of Malaya had successfully determined a list of common genomic copy number alterations that were involved in oral cancer progression through the analysis of accumulation of genetic changes that took place from the oral precursor lesion stage to the development of invasive tumour (Salahshourifar *et al.*, 2014). It has been reported that even though many OSCC emerge from OPMDs, OSCC generally experienced greater genomic gains and losses compared to OPMDs (Mortazavi *et al.*, 2014; Salahshourifar *et al.*, 2014). The hypothetical model of oral carcinogenesis is illustrated in Figure 2.1.

In the study conducted by Oral Cancer Research and Coordinating Center (OCRCC), Faculty of Dentistry in University of Malaya has identified the deletions of tumour suppressor genes were linked to the initial events in carcinogenesis as the loss of 3p14.2 and 9p21 were observed in low grade dysplasia and some cases of histologically normal epithelial cells (Salahshourifar *et al.*, 2014). FHIT gene that sits on 3p14.2 is a putative tumour suppressor gene that plays a role in the regulation of apoptosis and cell cycle (Croce *et al.*, 1999). Oral premalignant lesions with a deletion at 3p14.2 have always been categorized as high risk lesions, and the loss of heterozygosity in this gene is usually more prominent in patients that are exposed to environmental carcinogens (Croce *et al.*, 1999; Zhang *et al.*, 2012). On the other hand, 9p21 contains the CDKN2A suppressor gene

that encodes p16 and p14 proteins that in turn, regulate the p53 and RB1 cell cycle pathways (Pacifico & Leone, 2007). Though p53 is over-expressed in the early stage of dysplasia due to the inactivation of CDKN2A, this gene was found to be downregulated instead, as a result of genetic mutations in the later stage when OSCC develops. Therefore, inactivation of p53 could only be found in OSCC samples but not the corresponding dysplastic tissues (Shahnavaz *et al.*, 2000; Hsieh *et al.*, 2001).

In mild dysplasia to OSCC, gains in 8q22-q23 are common, while gains in the whole 8q arm may be an indicator of invasive OSCC (Noutomi *et al.*, 2006; Garnis *et al.*, 2009). Apart from this, one of the hotspot regions for high grade dysplasia is also inclusive of 8q24, which accommodates the myelocytomatosis (MYC) oncogene and other genes like Protein Tyrosine Kinase 2 (PTK2) and Lymphocyte Antigen 6 Complex, Locus K (LY6K), as well as 8q22 that contains the Low Density Lipoprotein Receptor-Related Protein 12 (LRP12) gene (Garnis *et al.*, 2004b). Besides chromosome 8, gain in 11q13 was also reported as a result from the literature by Salahsourifar *et al.* (2014). CCDN1 gene is also located in this region. Moreover, over-expression of EGFR as a result of amplification of 7p11.2 was also detected and had been said to be connected to the poor prognosis of OSCC as this alteration could only been found in high grade dysplasia (Sheu *et al.*, 2009; Ribeiro *et al.*, 2012). The next frequent alteration found was the gain in 3q which carries several genes related to the tumourigenesis such as TP63 and PIK3CA genes. Subsequently, over-expression of these genes increases the risk of cancer progression as they are responsible for regulation of cell cycle, apoptosis and metastasis (Melino, 2011; Su *et al.*, 2013).

In addition, when comparing with other study, it has been shown that deletions in the short arm of chromosome 3 is a potential biomarker for oral cancer progression as the non-progressive low grade dysplasia demonstrated 3p25.1-3p25.3 deletion whereas the progressive low and high grade dysplasia exhibited deletions in regions 3p25.3-p26.1, 3p25.1-p25.3, 3p24.1, 3p21.31-p22.3, 3p14.2 and 3p14.1 (Tsui *et al.*, 2009). Meanwhile, OSCC patients showed more than 90% of deletions in the previous chromosomal locations stated and the complete loss of the 3p arm indicated advanced tumour stage (Tsui *et al.*, 2009). Significant CNAs detected in lymph node metastatic tumours include high copy numbers in 7p12 (EGFR), 11q13 (FGF3/FGF4, CCND1, EMS1), 17q11.2 (THRA) and 20q12 (AIB1) (Chen *et al.*, 2004). In another study by Liu *et al.* (2006), 3q26.3 (PIK3CA) and 11q13 (FGF4) showed gain within the metastatic samples, but not with matched primary tumours.



Whole arm represents several chromosomal alterations on that specific arm and those highlighted in bold and underline have shown higher frequency.

Group I: clinically and histologically normal labial mucosa may manifest subtle genetic changes.

Group II: different OPMDs display varied histological appearances from hyperplasia to low grade dysplasia that may have some genomic instability.

Group III: OPMDs with severe histopathological changes such as high grade dysplasia or carcinoma in situ with accumulation of genetic changes.

Group IV: clinical appearance of histologically proven OSCC as an ulcer or proliferative growth inside the oral cavity

Group V: advanced stage of OSCC with lymph node metastasis.

Figure 2.1: Hypothetical model of oral carcinogenesis. Putative sequence of genetic alterations that drive OSCC from normal appearing epithelial cells. The image is reprinted with kind permission of the publisher, (Salahshourifar *et al.*, 2014).

2.11 "Omic" profiling

In 2001, the International Human Genome Sequencing Consortium (2004) successfully identified a total of 20,000 to 25,000 protein-coding genes. The completion of the human genome sequencing project resulted in the advancement of high throughput technologies for "omic" profiling. This profiling technique allows large scale discovery of aberrant genes and molecular pathways in cancer, thus contributing to a better understanding of cancer pathogenesis (Baak *et al.*, 2003; Nagaraj, 2009). With the introduction of high throughput technologies combined with sophisticated bioinformatic tools for complex data analysis, comprehensive and systematic "omic" profiling has been developed to examine candidate genes interaction within biological pathways and networks in cancer (Martin & Nelson, 2001). Due to the complex nature of cancer, "omic" technologies such as genomics (the study of the human genome), transcriptomics (gene expression) and proteomics (the analysis of the protein complement of the genome) take a central place in discovering new biomarkers pivotal for developing new diagnostic and therapeutic strategies (Garnis *et al.*, 2004a). In addition, specific cellular functions and their interaction to produce cancer phenotypes can be predicted or interpreted based on results from high throughput analysis (Nagaraj, 2009). One of the genomic technologies is the array CGH as this technology allows screening of copy number alterations in a single experiment (Marquis-Nicholson *et al.*, 2010).

2.11.1 Array CGH

Array comparative genomic hybridization is a high throughput array which the array is spotted with elements produced by DNA sequences that were used to replace the metaphase chromosomes of the conventional CGH technique as the target for analysis (Lucito *et al.*, 2003). This technology provides new information on genomic copy number alterations which is valuable in classifying cancer subtypes (Kallioniemi, 2008). In this technique, genome database is used to design probes/clones sequence to be spotted onto glass microscope slides (array) to detect alterations in specific genes. Therefore, this technique is being increasingly utilized to determine and pinpoint candidate genes that are drivers of cancer and genetic disease pathogenesis (Inazawa *et al.*, 2004; Lockwood *et al.*, 2006).

Array CGH can overcome various limitations reported for conventional CGH (Inazawa *et al.*, 2004; Pinkel & Albertson, 2005; Kallioniemi, 2008). The superiority of the current array CGH in cytogenetic methods includes higher resolution, higher throughput, possibilities for automation, robustness, simplicity, high reproducibility and precise mapping of alterations (Inazawa *et al.*, 2004; Pinkel & Albertson, 2005; Kallioniemi, 2008). The higher resolution of array CGH can be ascribed to the usage of smaller sized DNA probes while the level of resolution is determined by the size of nucleic acid targets and the density of coverage over the genome (Bejjani & Shaffer, 2006). In cancer research, the ultimate goal of array CGH analyses is to allow precise localization of specific genetic modifications associated with tumour progression, therapy response, or patient prognosis. This method facilitates the discovery of predictive and prognostic biomarkers for tumour progression in cancer (Kallioniemi, 2008; Shinawi & Cheung, 2008). On the

other hand, the limitations of array CGH include high cost as well as the inability of the technique to detect balanced rearrangements in translocations and inversions, as only copy number imbalances relative to other DNA regions can be detected.

2.11.2 OSCC associated CNAs

Array comparative genomic hybridization identifies common CNAs and their frequency in OSCC samples as described by Salahshourifar *et al.* (2014). This study incorporates previous studies on OSCC tumours using array CGH, where the tumour sites contained only in the oral cavity from tongue, gum, hard palate, soft palate, floor of mouth and buccal mucosa and excludes studies on cell lines, Single nucleotide polymorphisms (SNPs) and customized array CGH (Table 2.2). Based on intensive literature review, the most commonly reported CNAs were as follows: gains 3q, 5p, 7p, 8q, 11q and 20q; and losses 3p, 8p and 18q whereby CNA of gain in 8q as the most commonly reported amongst all OSCCs. Further study revealed that genes in 8q24: *PTK2*, *LY6K* and *MYC* and 8q22: *LRP12* may be related to tumour inducing genes in oral cancer.

The second most common CNA was detected in the gain in 11q13 which harbors genes that are actively involved in tumour progression, invasion and metastasis. In the 11q13 region, *CCND1* was found to be over-expressed in clinical samples with high grade dysplasia and was associated with patient survival (Garnis *et al.*, 2004a; Tsui *et al.*, 2009). *CCND1* is amplified in various cancers, is over-expressed in oral epithelial dysplasia in transgenic mice, and was discovered to be a proto-oncogene controlling the G1/S transition in cell cycle (Wilkey *et al.*, 2009; Musgrove *et al.*, 2011). Furthermore, *CCND1* is highly involved in tumourigenesis along with other cancer genes such as *EMS1* and *ORAOV1* in

the 11q13 region (Huang *et al.*, 2006). Apart from these regions, amplification in 7p11.2 was high in OSCCs leading to over-expression of *EFGR* which was highly expressed in high grade dysplasia cases associated with poor prognosis (Sheu *et al.*, 2009; Ribeiro *et al.*, 2012). High rate of transformation from OPMD to OSCC was associated with high *EFGR* copy number (Taoudi Benchekroun *et al.*, 2010). Therefore, it was concluded that the over-expression of both *CCND1* and *EFGR* was highly associated with the development of OSCC and high risk OPMDs (Tsui *et al.*, 2009).

The second most common chromosome aberration was also detected in the gain in 3q region which consisted of *TP63*, located specifically in 3q26. *TP63* gene is a tumour protein highly associated in the transformation of epithelial dysplasia to OSCC and is significantly linked to poor prognosis in patients (Matsubara *et al.*, 2011). *TP63* is usually expressed in normal epithelial tissues, however over-expression of this gene exhibits severe dysplasia (Das *et al.*, 2010; Su *et al.*, 2013). The gain in 3q leads to the subsequent over-expression of *TP63* and *PIK3CA*, whereby higher copy number is highly associated with increased prognosis. The tumour suppressor role of *TP63* involves in cell cycle regulation, apoptosis, metastasis and tumourigenesis with the interaction with p53 is well defined (Melino, 2011; Su *et al.*, 2013).

Next, the high losses were observed in the 3p, 8p and 9p regions, but losses in 3p and 9p were associated with early tumourigenesis. Advanced stages of OSCC were based on the chromosomal instability that extends to the whole chromosome arm and genetic heterogeneity (Salahshourifar *et al.*, 2014).

In 2011, in order to complement the exome sequencing data, Agrawal et al. (2011) had performed a genome wide profiling using the SNP microarray on 42 HNSCC that corresponded to the 25 samples involved in the exome sequencing study. The integration between these data revealed that most of the frequently mutated genes that undergone LOH mutation like TP53, CDKN2A and NOTCH1 were found to be deleted in chromosome 17p13.1, 9p21.3 and 9q34.3 respectively. Apart from that, the genome wide profiling from this study also identified chromosome 11q, 3q and 7p were found to be amplified in the HNSCC samples and the identified oncogenes that mapped in these regions were CCND1, P1K3CA and EGFR, respectively. Another genome wide profiling using similar microarray technology in Pickering et al. (2013) study has identified more than 50% (n = 40) of OSCC samples were experienced chromosome amplification in 8q, 3q and chromosome deletion in 3p, 8p and 18p. Deletion of chromosome 8p in this study was found to be associated with extracapsular spread and poor prognosis. Besides, the integrative analysis with gene expression data revealed that amplification of chromosome 11q13 was associated with over-expression of CCND1.

Recently, the cancer genome atlas consortium (TCGA) (2015) and International Cancer Genome Consortium (ICGC) (2013) has identified the most frequent amplifications were found in chromosome 3q, 5p, 7p, 8q and 11q whereas the most frequent deletions were found in chromosome 3p and 8p in OSCC patients. In TCGA, this consortium has identified the dual amplification of chromosome 11q13 and 11q22 as one of the CNAs that characterized HPV negative OSCC with the genes harboured in this loci being CCND1,

FADD and CTTN and BIRC2, YAP1 and MMP family genes implicated in cell death/NF-kB and Hippo pathways (TCGA, 2015).

These CNAs also identified in Vincent-Chong et al. (2013) studies that using an ultra-dense array CGH technology with array slides contains 974,016 probes for discovering CNAs within 45 OSCC samples in Malaysia. This study has have identified that the most frequently amplified region, 8p11.23-p11.22 contained several ADAM family genes (ADAM9, ADAM5P and ADAM32). Interestingly, almost the entire p-arm of chromosome 8 was amplified. The second most frequently amplified CNA, was a novel genomic amplification on chromosome 7q34, and contains the MGAM gene, which was present in 78% of OSCC samples. This study has identified significant regions in the OSCC genome that were amplified which results in consequent over-expression of MGAM and ADAM9 genes. Combining the finding of the precision, incidence (correlating to selective retention pressure of the CNA) and increase gene expression, MGAM could be a significant gene that drives OSCC development that may be utilized as biological markers for oral cancers.

Table 2.2: Frequent CNAs (amplifications and deletions) in OSCC. Adapted from (Salahshourifar *et al.*, 2014)

Gain/Loss	Chromosomal region	CNAs associated genes
Gain	3q27-q29 3q26 3q29 3q27.2-3q27.3 3q24-q29 3q24-q25 3q26-qter 3q25.31-q36.3 3q21.3-q29	*TP63, TERC, ZASC1, EPHB3,BCL6, TM4SF1, SOX2, CLDN1, SCCRO, PIK3CA*
Gain	5p13 5p15.33 5p13.1-p13.2 5p15 5p15.33-p11 5p13.2 5p13.1-p15 5p15.33	*TERT, RAD1, SKP2, SEC6L1*
Gain	7p12.3-p12.1 7p11 7p22.3-p11.1 7p11.2 7p12-p11.2 7p21.1 7p22.3 7p12.3-p12.1 7p11.2-22.3	*FSCN1, IL6, EGFR, GNA12*

Table 2.2, continued

Gain/Loss	Chromosomal region	CNAs associated genes
Gain	8q11.1-q24.4 8q24.12-q13 8q24.11-q24.13 8q24.23-q24.3 8q24 8q11.1-q24.4 8q22-23 8q 8q24.1-q24.2 8q21.1-24.3 8q11.1-q24.3	PRKDC, YWHAZ, LRP12, RAD21, EXT1, MYC, NDRG1, PTK2, LY6K
Gain	11q11 11q23.3-q25 11q13 11q13.3 11q13.5 11q 11q13.3 11q13.1 11q13.1-q13.3	CCND1, ORAOV1, FGF19, FGF4, FGF3, FADD, PPFIA1, EMS1, SHANK2, TPCN2, OCIM, BIRC3
Gain	20q11.21-q13.33 20q13 20q12 20q 20q13.33 20q13.3	AIB1, BCL2L1, MMP9, SNAI1, BMP7

Table 2.2, continued

Gain/Loss	Chromosomal region	CNAs associated genes
Loss	3p14.2 3p12-p13 3p24.3 3p24.2-p24.3 3p21-3p12 3p14.2 3p14-p21 3p 3p21.3 3p22 3p14 3p26.3 3p11.1-3p26.3	*FANCD2, VHL, TIMP4, PPARG, XPC, RARB,TGFBR2, MLH1,DLEC1, CSRNP1, CTNNB1, RASSF1, WNT5A, FHIT*
Loss	8p23.2 8p32 8p 8p11.23-p23.2	*PCM1, CSMD1, LZTS1*
Loss	9p21.3 9p21 9p 9p21.3	*CDKN2A, MTAP, CDKN2B*

CHAPTER 3: METHODOLOGY

3.1 Study design

A cross-sectional study was conducted where genome-wide profiling of 75 cases of OSCC was carried out using array CGH and 66 OSCC samples which overlapped with 75 array CGH samples was carried out using qPCR copy number assay to determine and validate the CNAs of OSCC. The samples for array CGH and qPCR consisted of gDNA of fresh-frozen OSCC tissues.

The gene(s) that mapped within the frequently amplified and deleted regions were subjected to pathway and network analysis using Ingenuity Pathway Analysis (IPA) software. The mRNA of the identified putative amplified genes involved with network signalling associated with cancer were determined using qPCR using cDNA from fresh-frozen OSCC and non-cancer oral mucosal tissues.

Protein expression of the identified amplified genes was determined using immunohistochemistry (IHC) staining of formalin fixed paraffin embedded (FFPE) tissues of non-cancer oral mucosa, OSCC and oral epithelial dysplasia (OED). The FFPE tissues were from tissue microarray (TMA) blocks consisting of five non-cancer or non-dysplasia, 15 OEDs and 103 OSCCs. The roles of the putative candidate gene in terms of cell proliferation, apoptosis, migration and invasion was investigated using the siRNA technique in three different OSCC cell lines derived from tongue, cheek and gum SCC

using MTT assay, caspase 3/7 luciferase assay, wound healing assay, transwell migration and transwell invasion assay. The workflow of this study is illustrated in Figure 3.1.

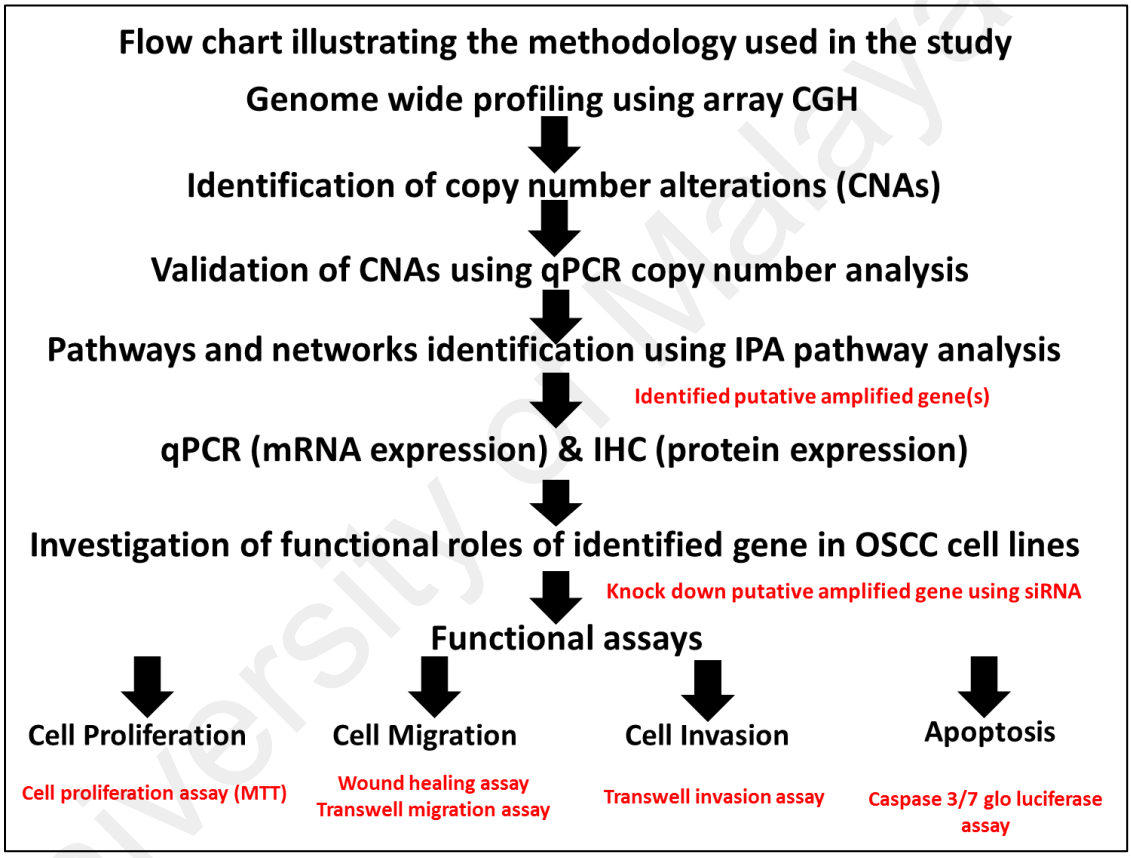


Figure 3.1: Project workflow.

3.2 Sample types

Three types of sample were involved in this study namely:

1. Fresh-frozen tissues obtained from the Malaysian Oral Cancer Database and Tumour Bank System (Zain *et al.*, 2013) coordinated by Oral Cancer Research and Coordinating Centre (OCRCC).
2. FFPE tissues arranged as tissue microarrays (TMAs) obtained from OCRCC archives of TMA blocks.
3. Oral cancer cell lines (from Cancer Research Malaysia) derived from OSCC tissues.

The OSCC site was classified according to the anatomical subsites of the International Classification of Disease (ICD-10), a coding system that was developed by World Health Organization (WHO). The anatomic subsites of the oral cavity are lips, floor of mouth, oral tongue (anterior two-thirds of tongue and excluding base of tongue), buccal mucosa, gingivae, hard palate, and retromolar trigone. In this current study, the tongue cancers were grouped as a single entity due to tongue cancers had the highest prevalence at 220 per 100,000 population compared to other subsites within the oral cavity in Malaysia (Omar & Tamin, 2011). Apart from that, tongue cancers are characterized by a more aggressive biological phenotype, with a high-propensity of cervical lymph node metastasis that might be reflected at their mutational and molecular level (Thangaraj *et al.*, 2016). The American Joint Committee on cancer staging criteria was used for tumour staging (Sobin *et al.*, 2011). All the OSCC samples that were included in this study were histologically confirmed OSCCs, where patients had not received prior therapies and the non-cancer individuals, were those who did not have a history of any other cancers including oral cancer. This study was approved by

the Medical Ethics Committee (MEC), Faculty of Dentistry, University of Malaya with the MEC code no: DF0306/ 001/(L) and DF OS1007/0048(P).

3.3 Sample selection criteria

Sample selection for all specimens was based on the following inclusion and exclusion criteria below -

Inclusion criteria:

1. Samples (fresh-frozen and FFPE tissues) histologically confirmed to be OSCC and non-cancerous oral mucosa for control.
2. Fresh-frozen tissues of the primary tumour consisting of epithelial tumour cells with evidence of more than 70% of OSCC tumour cells.
3. Fresh-frozen tissues of the non-cancer oral mucosa consisting of more than 70% non-cancer surface epithelial cells (most of the underlying connective tissue was macro-dissected to achieve this).

Exclusion criteria:

1. Oral cancers (for both fresh-frozen and FFPE tissues) which are pathologically diagnosed as other cancers (not OSCC).
2. Fresh-frozen tissues < 70% OSCC tumour cells.
3. Fresh-frozen tissues < 70% non-cancer surface epithelial cells.

3.4 Laboratory procedures

3.4.1 DNA and RNA extraction from tissue samples

Frozen tissue specimens were mounted in optimal cutting temperature (OCT), sectioned and stained with haematoxylin and eosin (H&E) for histological assessment where the diagnosis was confirmed. The percentage of tumour cells was gauged under the microscope by oral pathologists (Prof Rosnah Zain, Dr Thomas Kallarakkal, and Dr Anand Ramanathan). For any tissues that did not reach a 70% tumour content, macro-dissection of the OCT embedded tissue was carried out to obtain ≥ 70% tumour tissues for DNA and RNA extraction. All tumour tissues for test genomic DNA (gDNA) and RNA were isolated by using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and RNeasy micro kit (Qiagen, Hilden, Germany), respectively according to the manufacturer's instructions. The quality (A260/A280) and quantity (ng/µl) of gDNA were determined using a spectrophotometer (Nanodrop ND-2000, Thermo Scientific, MA, USA). The integrity of RNA was accessed using the Agilent Bioanalyzer-2100 (Agilent Technologies, CA, USA) to determine the quality of the extracted RNA from the samples. The Agilent Bioanalyzer-2100 software (Agilent Technologies, CA, USA) can detect the ratio of the 18S and 28S ribosomal RNA (rRNA) and generate the RNA integrity number (RIN). This RIN is classified from 1 to 10, with 1 being the most degraded total RNA and 10 being the most intact total RNA. Detailed protocols used for DNA and RNA extraction are included in Appendix A and B, respectively.

3.4.2 Array CGH

A total of 75 cases of OSCC cases were selected from the MOCDTBS for array CGH study. Detailed information on the OSCC frozen tissues used for array CGH is attached in Appendix C. Genome-wide screening with customizing oligonucleotide array CGH (8x60k) (Agilent Technologies, CA, USA) was performed according to the manufacturer's instructions (version 5.0, June 2007) by Oxford Gene Technology, Oxford, UK. Enzymatic digestion was replaced with a heat defragmentation step during sample preparation. This was followed by sample labelling, probe purification, microarray hybridization, washing and scanning (samples were processed by Oxford Gene Technology). For each array CGH experiment, 1.5 µg of DNA (gDNA) from each fresh-frozen tumour sample (test sample) and commercially gender matched pooled blood gDNA sample (Promega Corporation, WI, USA) were taken and then labelled with fluorescence Cy5 and Cy3 dyes in dye-swap experiments using the CytoSure Genomic DNA labelling kit (Oxford Gene Technology, Oxford, UK), respectively. Once the labelling reaction was completed, labelled test and reference test samples were purified and concentrated using Microcon YM-30 filters (Merck Millipore, MA, USA) and then mixed together. This mixture was then subjected to probe denaturation and pre-annealing with Cot-1 DNA. Hybridization was performed at 65^{o} C for 40 hours with a constant rotation at 20 rpm. After hybridization, slides were washed according to the manufacturer's instructions and scanned immediately using a DNA Microarray Scanner (Agilent Technologies, CA, USA). The signal intensities were generated using Feature Extraction software, version 10.7.3.1 (Agilent Technologies, CA, USA) to produce a text file per array. The data from the text file were segmented using a modified Circular Binary Segmentation (CBS) algorithm (Venkatraman & Olshen, 2007). The CNAs were identified using CytoSure Interpret software version 4.2.5 (Oxford Gene

Technology, Oxford, UK) by applying $\log_2$ intensity ratios of sample to reference (Cy3/Cy5: log2-ratios above 0.3 for amplifications and below -0.6 for deletions). Data obtained was extracted from the scanned images. The start and end points of the CNAs were analyzed against the version of the human genome assembly (hg19/GRCh37). The result was an integrated dataset consisting of a list of cytobands with median start and end positions according to the latest human genome assembly database. Classification of the CNAs was conducted without any limit in size. Therefore, the CNA regions that are gene-rich, most likely would be pathogenic containing a specific region of interest. Overlapping regions were resolved by utilizing the lowest start and highest end position. The numbers of amplifications and/or deletions were calculated by quantifying the number of redundant and overlapping regions for each cytoband. Finally, the frequency was calculated, and cytobands were ranked according to their amplification and/deletion percentages. An arbitrary frequency threshold of 5% was used to define if a CNA could be of importance and genes in regions altered in at least 5% of specimens were used for pathway and network analysis as described below.

3.4.3 Functional analysis

The gene that mapped within the regions of the CNAs that were altered in at least 5% of the specimens were subjected to gene networks and significant biological functions (diseases, molecular and cellular functions) analysis using IPA software (Ingenuity Systems, CA, USA). In the IPA software, the default setting was set to map CNA associated genes to the knowledge base which is a reference set of direct and indirect relationships. Next, relevant input to the gene list such as the molecular networks and biological functions were generated by the software algorithm. The p-value which indicates the probability of the genes in a network generated based on random chance and ranking by available networks in the Ingenuity database. The significance of each biological function was determined with right-tailed Fisher's exact test with $p < 0.05$ significance. From the generated network signalling, putative novel oncogenes, namely MMP13 and ISG15 were prioritized for further evaluation and characterization in OSCC by quantification of mRNA and protein expression levels as their clinical impact in oral cancer is poorly understood and literature suggests a high probability of involvement in tumourigenesis. These genes were found to harbor rare amplicons (chromosome 1p36.33 and 11q22.2), however, it has been hypothesized that even rare amplicons would be informative regarding genes crucial in tumor development as the expression may be altered by either copy number changes or other mechanisms by disrupting the upstream or downstream genes in different mechanisms that control a particular pathway (Snijders *et al.*, 2005). Therefore, in order to test this hypothesis whether ISG15 is a driver gene in OSCC (as MMP13 roles in oral tumorigenesis has been studied previously). Therefore, ISG15 was selected for further downstream analysis which is to determine its gene and protein expression in OSCC samples as well as its functional roles via *in vitro* study using OSCC cell line models based

on its involvement in the oncogenic network (cell death and survival, cellular movement, cellular development).

3.4.4 Copy number analysis by the TaqMan PCR assay

DNA was extracted from normal samples/tumour tissues with ≥ 70% tumour cell content using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol (Appendix A). Copy number analysis was performed on 66 OSCCs according to the manufacturer's protocol. The detail information on the OSCC frozen tissues used for Taqman PCR assay is attached in Appendix D. Briefly, each gDNA was analyzed in triplicates by duplex TaqMan real-time polymerase chain reaction assays. The commercially male and female pooled blood gDNA sample (Promega Corporation, WI, USA) served as calibrator controls. Copy number analysis was done using TaqMan Copy Number Assay: LRP12 (Hs01987319_cn), FSCN1 (Hs03631914_cn), EGFR (Hs02309320_cn), CCND1 (Hs02226007_cn), CHL1 (Hs02163529_cn), TPM2 (Hs01060645_cn), CLPTM1L (Hs01133209_cn), CSMD1 (Hs03683117_cn) (Applied Biosystems, Foster City, CA, USA). PCR was done in a total volume of 20 µl consisting of 4 µl of genomic DNA(5 ng/µl), 10 µl of 2× TaqMan® Genotyping Master Mix (Applied Biosystems, CA, USA), 1 µl of 20X TaqMan Copy number assay, 1 µl of 20X TaqMan copy number reference assay (RNAse P) and 4 µl of nuclease free water. Quantitative PCR was performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems, CA, USA) using the manufacturer's PCR conditions as follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing for 60 seconds at 60°C.

The values of copy number for each sample were normalized using RNAase P as a reference control with 2 copies in the human genome. Copy number was quantified using the equation 2 x ($2^{-\Delta\Delta Ct}$), comparative CT (ΔΔCT) relative quantitation method (Livak & Schmittgen, 2001). Target and reference assays that were used for copy number calculation were derived from the mean of triplicate, RNase P and the calibrator samples. The calculated relative quantity was multiplied by a base copy number of 2 to obtain the copy number value. The values less than one and more than 2.0 were considered as deletion and amplification, respectively (Bronstad *et al.*, 2011; Lopez-Nieva *et al.*, 2012).

3.4.5 Real time quantitative PCR assay for gene expression

Selected identified genes (MMP13 and ISG15) based on network analysis were validated with an independent set of 68 OSCC samples for MMP13 and 64 OSCC samples for ISG15 using qPCR. The detailed information on the OSCC frozen tissues used for gene expression qPCR study of MMP13 and ISG15 are attached in Appendix E and F, respectively. Total RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). A detailed protocol used for High Capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) is included in Appendix G. Quantitative-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA) in triplicate on cDNA of the samples using TaqMan Fast Universal PCR master mixture (Applied Biosystems, CA, USA). The TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) used were MMP13 (Hs00233992_m1) and ISG15 (Hs00192713_m1). All qPCRs were carried out according to the manufacturer's protocol (Applied Biosystems). The relative quantification or fold change (RQ) of all genes was calculated using the $2^{-\Delta\Delta CT}$ method using 7500 Fast System

SDS Software 1.3.1 (Applied Biosystems, CA, USA). The housekeeping gene (GAPDH) was used as an endogenous control while the cDNA from normal oral mucosa tissue which was used to normalize test samples (OSCC).

3.4.6 Tissue microarray

Tissue microarray (TMA) of 1.0 mm core size had been constructed as described previously (Kononen *et al.*, 1998) using a semi-automatic Tissue Arrayer Minicore (Alphelys, SAS, France). All OSCC FFPE blocks and the respective 5 µm H&E stained slides were reviewed to identify and mark out the representative tumour areas by oral pathologists. Approximately, three to six cores from the selected areas of donor blocks were transferred to the recipient paraffin blocks. The completed recipient paraffin blocks also known as TMA were incubated overnight at 37°C and 4 µm thick sections were sectioned using rotary microtome and placed onto the poly-L-lysine slides.

3.4.7 Immunohistochemistry and scoring system

3.4.7.1 Immunohistochemistry staining

The immunohistochemistry (IHC) was performed on 4 µm thick FFPE sections using the Dako Real EnVision Detection System and Peroxidase/DAB+ (Dako Corporation, CA, USA) according to the manufacturer's protocol. Briefly, FFPE sections were deparaffinized in xylene and rehydrated in ethanol series increasing from absolute ethanol, 80% ethanol and 70% ethanol. Antigen retrieval was carried out using an electric pressure cooker (110ºC, 20 minutes) in 10 mM citrate buffer (pH 6.0). The sections were immersed in blocking solution (Dako Corporation, CA, USA) for blocking the endogenous peroxidase activity for 10 minutes at room temperature followed by washing with

phosphate-buffered saline (PBS) (pH 7.4) plus 0.1% Tween 20. Sections were then incubated with the optimized concentration of 8 µg/ml of monoclonal anti-MMP13 (MAB511, R&D Systems, Inc, Heidelberg, Germany) overnight at 4°C and polyclonal rabbit antibody anti-ISG15 (HPA004627, Sigma-Alrich, MO, USA) at the optimized dilution factor of 1:100 for 60 minutes at room temperature. After washing with PBS buffer, sections were incubated with the peroxidase labeled secondary antibody from the Envision kit for 45 minutes for the immunoreactivity performances. Finally, sections were stained with 3'3 diaminobenzidine substrate chromogen, (Dako Corporation, CA, USA) counterstained with Mayer's haematoxylin, dehydrated and mounted with a xylene-based mounting medium. Ovarian and hepatocellular cancer tissue were used as positive control for MMP13 and ISG15, respectively. Negative control was performed using the same condition; except that the primary antibody was replaced with PBS on OSCC section that was known to stain positively with MMP13 and ISG15 primary antibodies. The detail information on the FFPE OSCC used for IHC protein expression study of MMP13 and ISG15 are attached in Appendix H and I, respectively.

3.4.7.2 Scoring of immunohistologically stained samples

Digitized immunostained TMA spots were analyzed and scored by two oral pathologists (Dr Anand Ramanathan and Dr Thomas George Kallarakkal) independently based on a semi-quantitative scoring system using TMA software module 1.15.2 (3DHISTECH, Budapest, Hungary). The intensity scores were quantified using the following scores: negative = 0; weak = 1; moderate = 2 and strong = 3. The proportion of immune-positive cells was quantified as follows: 0 = negative; 1 = ≤10%; 2 = 11-50%; 3 = 51-80% and 4 = ≥ 80% of positive cells. The final immune-reactive score was determined

by multiplying the intensity and the proportion scores of the stained cells to obtain an immune-reactive score ranging from 0 to 12 (Bektas *et al.*, 2008; Liao *et al.*, 2009). Cores with discrepant scores were discussed by both pathologists to achieve a consensus to derive the final score. The mean of consolidated immune-reactive scores for each case was recorded.

3.4.7.3 Selection of cut-off score for ISG15 and MMP13 protein expression

The clinico-pathological parameters were first dichotomized as following: lymph node metastasis (no vs yes), tumour staging (early vs advanced), tumour sizes (T1 and T2 vs T3 and T4) and survival status (alive vs dead). Receiver operating characteristic (ROC) curve analysis was used to determine the best cut-off score for MMP13 and ISG15 protein expression to each of dichotomized clinico-pathological parameters using 0, 1 criterion (Zlobec *et al.*, 2007). For MMP13 and ISG15 immunoreactive scoring, the sensitivity and specificity of each score were plotted to generate various ROC curves against with clinico-pathological parameters. The score which was closest to the point with maximum sensitivity and specificity was selected as the cut-off value. The immune-reactive scores were divided into high and low MMP13 and ISG15 expression where low expression was the scores below or equal to the cut-off value, while high expression were the scores above the cut-off value.

3.5 Cell culture and siRNA transfection

The OSCC cell lines (ORL-48T, ORL-188T and ORL-204T) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) F12 (Gibco, Life Technologies, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C. Detailed information of the OSCC cell lines used for the *in vitro* study is attached in Appendix J.

For transfection, cells were seeded in 6 well plates at 2.0×10^5 cells per well at the time of standard transfection. Knockdown of ISG15 genes was established by a standard transfection using ON-TARGET plus SMARTpool siRNAs that directed against ISG15 according to the manufacture's protocol (Dharmacon, PA, USA) using DharmaFECT 1 siRNA Transfection reagent, with final siRNA concentration for 48 hours. ISG15 gene was targeted with a SMARTpool that comprised four different siRNAs. The four different targeting sequence are ON-TARGETplus SMARTpool siRNA J-004235-21, ISG15 (Target Sequence: GCGCAGAUCACCCAGAAGA); ON-TARGETplus SMARTpool siRNA J-004235-22, ISG15 (Target Sequence: GCAACGAAUUCCAGGUGUC); ON-TARGETplus SMARTpool siRNA J-004235-23, ISG15 (Target Sequence: GGCUGACCUUCGAGGGGAA) and ON-TARGETplus SMARTpool siRNA J-004235-24, ISG15 (AAAGGCUGUUGUAAAGAGA). To ensure the transfection process and reagent does not alter gene expression level and phenotype, the non-targeting control siRNA is used. As the negative control (siRNA-NC), D-001810-01-05, ON-TARGET plus non-targeting control siRNA was used. The optimized final concentration used for the siRNA for ISG15 knockdown of ORL-48T, ORL-188T and ORL-204T is 25 nM, 25 nM and 50 nM respectively. The knockdown effect was checked by Western blotting.

3.6 Protein extraction and western blot analysis

Cell pellets were lysed in radioimmunoprecipitation assay (RIPA) buffer (89900, Pierce Protein Biology, CA, USA) supplemented with a protease/phosphatase inhibitor cocktail (1861281, Pierce Protein Biology, LA, CA, USA). Protein concentrations were determined *via* Bradford assay (Biorad, CA, USA). Approximately 50 ug of protein was mixed with 5x Laemmli buffer and heated in 95°C for 15 minutes prior to resolution on 12% SDS PAGE using the Bio-Rad mini gel electrophoresis apparatus for an hour in transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol, pH 8.3). The resolved proteins were electrophoretically transferred to polyvinylidene fluoride membrane (PVDF) (Merck Millipore, MA, USA) in wet condition at 400 mA for an hour using the Bio-Rad mini gel transfer system. Membrane was then blocked with 5% skimmed milk/TBST (Tris-Buffered Saline and Tween 20) to prevent non-specific binding of antibodies to the membrane for an hour. The PVDF membrane was incubated with primary antibody of rabbit anti-human ISG15 (HPA004627, Sigma-Aldrich, MO, USA) at 1:1000 dilution. The PVDF membrane was then probed with the respective secondary antibodies conjugated with horseradish peroxidase (Rabbit anti-mouse IgG H&L (HRP), ab6728, Abcam, Cambridge, United Kingdom and Goat anti-rabbit IgG H&L (HRP), ab97051, Abcam, Cambridge, United Kingdom) at room temperature for an hour. Antibody binding was visualized using enhanced chemiluminescence (Merck Millipore, MA, USA). The membrane was scanned using the Odyssey scanner (LICOR, NE, USA). To normalize for loading, the PVDF membrane was probed with mouse anti-β-Actin antibody (1:5000; Sigma-Aldrich, MO, USA) monoclonal antibody for an hour at room temperature and processed as described above. Images were acquired on the ODYSSEY Sa Infrared Imaging System (LICOR, Nebraska, USA).

3.7 *In vitro* functional assay

3.7.1 Cell proliferation assay using MTT assay

In order to assess the effect of ISG15 knockdown on cell viability, an MTT assay was performed. All three OSCC cell lines namely ORL-48T, ORL-204T and ORL-188T, transfected either with non-targeting siRNA control or ISG15 siRNA were suspended (4.0 × 10^3 cells) in 500 µl of medium supplemented with 10% FBS, plated in 24-well microplates and cultured for 24, 48, 72, 96, 120 and 144 hours. Fifty microliters (µl) of 5mg/ml MTT (Calbiochem, CA, USA) was added and incubation was carried out to form formazan crystals. After 4 hours of incubation, 500 µl of each detergent agent (0.01M HCL of 10% SDS) was added in each of the well in 24-well plate. The absorbance was measured at 570 nm (A_{570}) and reference absorbance was 670 nm (A_{670}) using the Tecan Infinite M200 (Tecan, Zürich, Switzerland) plate reader. The cell viability is proportional to A_{570}-A_{670}.

3.7.2 Cell proliferation assay using impedance measurement with the xCELLigence System

Cell proliferation (cell index) was measured by the xCELLigence Real-Time Cell Analyzer (RTCA) instrument according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences). OSCC cells transfected either with non-targeting siRNA control or ISG15 siRNA were seeded in triplicate at 1.0 × 10^4 cells/well into 100 µl of media in the E-Plate 16 (Roche, cat# 05469830001, Basel, Switzerland) after baseline measurement. Each of the 16 wells on the E-Plate 16 contains an integral sensor electrode array, so that cells inside each well can be monitored and assayed. The attachment, spreading and proliferation of cells in terms of the cell index (arbitrary unit reflecting the

cell censor impedance) were measured every 30 minutes using the RTCA software 1.2 (Roche, Basel, Switzerland). Cell proliferation was monitored for 30 hours in triplicate. The cell doubling time was calculated from the exponential phase of the growth curve using the RTCA software 1.2 (Roche, Basel, Switzerland).

3.7.3 Cisplatin sensitivity assay

The MTT cell proliferation assay (Calbiochem, CA, USA) was used to measure the cell growth inhibition effects of cisplatin (Tocris Bioscience, MO, USA) in the OSCC cells. Briefly, OSCC cell suspensions (at 1.0×10^5 cells/ml) were transferred to 96-well plates in triplicate and incubated for 48 hours with different cisplatin (Tocris Bioscience, MO, USA) concentrations (ranging between 0 and 100 µM). Then, 20 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml) was added to each well and incubated for 4 hours. Fifty microliters of each detergent agent (0.01M HCl of 10% SDS) were added in each well of the 96-well plate to resolve the crystals. The absorbance was measured at 570 nm (A_{570}) and reference absorbance was 670 nm (A_{670}) using the Tecan Infinite M200 plate reader (Tecan, Zürich, Switzerland). The cell viability is proportional to A_{570}-A_{670}. The IC_{50} of the OSCC cells were determined from absorbance versus concentration curve using Compusyn software (www.combosyn.com). The half maximal inhibitory concentration ($IC_{50)}$ determined was used for the subsequent treatments for the OSCC cell lines.

In order to investigate the role of ISG15 in affecting the cisplatin sensitivity of OSCC cells, similar MTT assay experiments were performed using non-targeting siRNA control or ISG15 siRNA transfected OSCC cell suspensions. Briefly, 1.0×10^5 cells/ml were transferred to 96-well plates in triplicates and incubated for 48 hours with cisplatin in

complete medium with their respective IC_{50} for 48 hours. Then, 20 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml) was added to each well and incubated for 4 hours. Fifty microliters of each detergent agent (0.01M HCl of 10% SDS) was added to each well to resolve the crystals. The absorbance was measured at 570 nm (A_{570}) and reference absorbance was 670 nm (A_{670}) using the Tecan Infinite M200 plate reader (Tecan, Zürich, Switzerland). The difference in absorbance by the cisplatin was used as the measurement of cell viability, normalized to non-targeting control, which was considered 100% viable.

3.7.4 Apoptosis detection using Caspase-3/7

ORL-48T, ORL-204T and ORL-188T cells transfected either with non-targeting siRNA control or ISG15 siRNA were seeded at a density of 1.0×10^4 cells/well into a 96-well plate in triplicate and treated/non-treated with cisplatin in complete medium with their respective IC_{50} for 48 hours. Caspase-3/7 activities were measured using a luminescent Caspase-Glo 3/7, assay kit (Promega Corporation, WI, USA) following the manufacturer's instructions. Caspase reagents were added to triplicate in the 96-wells and incubated for an hour. The caspase activities were measured using luminescent detector from Tecan Infinite M200 plate reader (Tecan, Zürich, Switzerland).

3.7.5 Wound healing assay

Wound healing migration assay was performed using a 35 mm µ-Dish (ibidi GmbH, Munich, Germany). A total of 70 µl of 35,000 cells (ORL-48T, ORL-204T or ORL-188T) transfected either with non-targeting siRNA control or ISG15 siRNA were seeded into each chamber of the cell culture insert overnight. The next day, cells were treated with 10 µg/ml of Mitomycin-C (Nacalai Tesque Inc, Kyoto, Japan) for 2 hours at 37°C before the cell culture insert was gently removed with sterile forceps. The cultures were washed twice with PBS, then, a medium supplemented with 5% FBS was added into the 35 mm µ-Dish. The cells that migrated into the denuded area were captured with Leica DMI3000B light microscope after optimized wound closure duration (Leica Microsystems GmbH, Wetzlar, Germany) at 50x magnification. The areas were analyzed using the Wimasis WimScratch software (ibidi GmbH, Munich, Germany). Wimasis WimScratch is a new generation web-based image tool for cell migration analysis. Edge detection techniques can easily recognize the leading edge and the gap area. The results were expressed as percentages of scratched areas. The average percentage scratched areas of siRNA transfected cells were used for the measurement of cell migration, normalized to non-targeting control, which was considered 100% migration.

3.7.6 Transwell migration assay

Transfected OSCC cells either with non-targeting siRNA control or ISG15 siRNA were tested in transwell migration assay using cell culture inserts with PET membrane of 8 µm pore sizes, according to the manufacturer's protocol (BD Biosciences, MA, USA). Briefly, OSCC cells were cultured and serum starved for 24 hours. Cells were then harvested and suspended in serum free DMEM F12 media at a concentration of 3.0 x 10^5 cells/ml (ORL-204T and ORL-48T) and 5.0 x 10^5 cells/ml for ORL-188T. In the 24-well plate, inserts were placed into wells containing 500 µl of DMEM F12 with 10% FBS media that acted as a chemoattractant, and followed by 200 ul of serum starved cells were seeded onto the insert for 24 hours. Post 24 hour incubation, the non-migrated cells were first eliminated by scraping with wet cotton swabs, while the bottom of the membrane was fixed and stained with 0.1% crystal violet (Biocalchem, Darmstadt, Germany) in 20% methanol for 2 hours at room temperature. Membranes were washed with water to remove excessive stained and air dried. The membrane was then viewed and captured under the Leica DMI3000B microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 100x magnification. The number of stained cells were counted in four randomly chosen microscopic fields and averaged. The average number of stained siRNA transfected cells were used for the measurement of cell migration, normalized to non-targeting control, which was considered 100% migration.

3.7.7 Transwell invasion assay

Experiments were carried out in the similar manner as described in the transwell migration assay except that the inserts used were pre-coated with matrigel basement matrix from Biocoat Matrigel 24-well invasion chamber (BD, Biosciences, MA, USA). Briefly, OSCC cells were cultured and serum starved for 24 hours. Cells were then harvested and suspended in serum free DMEM F12 media at a concentration of 5.0 x 10^5 cells/ml (ORL-204T and ORL-48T) and 8.0 x 10^5 cells/ml for ORL-188T. In the 24-well plate, inserts were rehydrated with serum free DMEM F12 media for 2 hours before they were placed into wells containing 750 ul of DMEM F12 with 10% FBS media that acted as chemoattractant. This is followed by seeding 500 ul of serum starved cells onto the insert for 24 hours. Post 24 hour incubation, non-migrated cells were scraped by wet cotton swab, while the bottom of the membrane was fixed and stained with 0.1% crystal violet (Biocalchem, Darmstadt, Germany) in 20% methanol for 2 hours at room temperature. Membranes were washed with water to remove excessive stained and air dried. The membrane was then viewed and captured under the Leica DMI3000B microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 100x magnification. The number of stained cells were counted in four randomly chosen microscopic fields and averaged. The average number of stained siRNA transfected cells were used for the measurement of cell invasion, normalized to non-targeting control, which was considered 100% invasion.

3.8 Statistical analysis

The association between selected CNAs and clinico-pathological parameters were analyzed by chi-square test (or Fisher exact test where appropriate). The CNAs, mRNA and protein expression level of candidate genes were compared between OSCC and non-cancer tissues using the Mann-Whitney U test. The copy number of selected CNAs associated genes was classified into three groups, deletion (< 1.0), amplification (> 2.0 copies) and no change (> 1.0 and ≤ 2.0 copies). Using the best cut-off point based on the immunoreactive scores of the MMP13 and ISG15 for specificity and sensitivity as mentioned in section 3.4.7.3, the association between the protein expression of MMP13 and ISG15 with the clinico-pathological parameters were analyzed by chi-square test (or Fisher exact test where appropriate). Survival curves were plotted and compared by the log-rank tests using the Kaplan-Meier analysis. In addition, Cox regression analysis was conducted to evaluate the MMP13 and ISG15 expression as an independent prognostic factor. The significant differences between the non-targeting siRNA control and the siRNA ISG15 were determined by the two-tailed multiple Student's t-tests with Bonferroni correction following the Dunnett's test. All data from cell culture functional assays were represented as average ± standard error of the mean (SEM) of three independent experiments each performed with duplicate/ triplicate wells. All statistical analyzes were performed using the SPSS statistical package (SPSS version 12.0, IL, USA) and the p-values < 0.05 was considered significant.

CHAPTER 4: RESULTS

This study aimed to determine the CNAs in OSCC using array CGH and to determine the expression of candidate genes. A putative candidate gene was identified and further elucidated to explore its potential role(s) in oral tumourigenesis. The results are presented in accordance with the specific objectives.

Objective 1: To determine and validate the CNAs of OSCC using array CGH and qPCR copy number assay.

4.1 Copy number alterations (CNAs)

A total of 75 OSCCs samples was subjected to array CGH to determine CNAs. The socio-demographic and clinico-pathological parameters of the OSCC samples are listed in Table 4.1. Amplification and deletions were observed in 28 and 3 chromosomal regions respectively (Table 4.2). The frequency, size and genomic start and end points of the CNAs are shown in Table 4.2. Numbers of chromosomal regions with amplifications were more frequently observed than deletions in the entire dataset. The frequent CNAs were observed on multiple genomic regions, including amplifications on chromosome 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q, and deletions on chromosome 3p and 8p. Amplification in chromosome 8q22.3-q23.1 was the most common and was found in 18.7% of OSCCs (n = 75) whereas deletion in 3p21.31 was the most common and was found in 9.3% of all samples (9 out of 75 OSCCs) (Table 4.2). Chromosomes 3q, 8q and 11q had the highest number of changes

in copy number at different regions across the entire length of the chromosome (Table 4.2 and Figure 4.1A).

4.1.1 Association of the copy number alterations with clinico-pathological parameters

To determine whether any of the frequent CNAs was associated with clinico-pathological parameters (tumour subsites, tumour sizes, LNM and tumour staging), chi-square analysis was performed. Results showed that amplification of chromosome 7p was significantly associated with tumour sizes (T1-T2: 31.1% vs T3-T4: 70%, p = 0.001) and tumour staging (early stages: 26.9% vs advanced stages: 57.1%, p = 0.013). Similarly, amplification of chromosome 8q, 11q and loss of 8p were found to be significantly associated with tumour staging (early stage: 15.4% vs advanced stages: 51.1%, p = 0.003), (early stages: 7.7% vs advanced stages: 28.6%, p = 0.036) and (early stages: 11.5% vs advanced stages: 38.8%, p = 0.014), respectively. There were also greater amplifications of chromosome 9p in OSCC patient with lymph node metastasis and the relationship was found to be statistically significant (LNM negative: 5.3% vs LNM positive: 24.3%, p = 0.020). As amplifications of chromosome 7p, 8q, 9p and 11q were significantly associated with clinico-pathological parameters, therefore the combination of these CNAs (chr7p+8q+9p+11q) was formulated as a genetic signature. Interestingly, the amplification of ≥ 1 of these CNAs within the genetic signature were found to be associated with tumour sizes (T1-T2: 55.6% vs T3-T4: 83.3%, p = 0.012), lymph node metastasis (LNM negative: 52.6% vs LNM positive: 81.1%, p = 0.009) and tumour staging (early stages: 42.3% vs advanced stages: 79.6%, p = 0.001) (Table 4.3 and Figure 4.1B).

Table 4.1: The socio-demographical and clinico-pathological parameters of the 75 OSCCs that involved in array CGH study.

Variables	Category	No. of patients (%)
Total		75
Gender	Male	26 (34.7)
	Female	49 (65.3)
Age (years)	< 45	12 (16.0)
	≥ 45	63 (84.0)
Smoking	No	52 (69.3)
	Yes	23 (30.7)
Drinking	No	64 (85.3)
	Yes	11 (14.7)
Betel quid chewing	No	40 (53.3)
	Yes	35 (46.7)
Tumour site	Tongue	24 (32.0)
	Non-tongue*	51 (68.0)
Tumour size	T1-T2	45 (60.0)
	T3-T4	30 (40.0)
Lymph node metastasis	Negative	38 (50.7)
	Positive	37 (49.3)
pTNM Staging	Early stage	26 (34.7)
	Advanced stage	49 (65.3)
Overall survival	Range	1-114 months
	Median	21.0 months
	Mean	26.24 months

*Buccal mucosa, gingiva, lip, floor of mouth, palate

Table 4.2: The amplified and deleted regions detected in 75 OSCC samples.

Cytoband	Median start position	Median end position	CNAs (AMP/DEL)	Number of sample	Percentage % (n=75)
8q22.3-q23.1	102681371	109392898	AMP	14	18.7
7p11.2	54033427	56399307	AMP	12	16
8q12.1	57356305	61290015	AMP	11	14.7
5p13.2-p13.1	34263518	42496863	AMP	11	14.7
9p21.1-p13.3	30940311	35689407	AMP	11	14.7
8q21.13-q21.2	48435432	57172822	AMP	10	13.3
7p22.1-p11.2	7091279	55728080	AMP	9	12
8q11.21-q12.1	48435432	102647978	AMP	9	12
5p13.3-p13.2	31085740	34171734	AMP	9	12
11q13.1-q13.2	63411714	67465752	AMP	9	12
8q23.1-q24.11	48435432	51961330.5	AMP	9	12
8q24.13	48435432	146301585	AMP	8	10.7
8q24.3	49084980	54788562	AMP	8	10.7
3q13.32-q21.2	117659990	125404921	AMP	7	9.3
8q21.2-q21.3	49084980	52283007	AMP	7	9.3
7p22.3-p22.1	16324	5554669	AMP	7	9.3
5p13.1	38651455	40760663	AMP	7	9.3
11q13.3-q13.4	69592775	71296836	AMP	7	9.3
3q25.31-q26.1	156865802	162501514	AMP	7	9.3
3q27.3-q28	186822642	189711307	AMP	7	9.3
8q24.12-q24.13	57804398	61290015	AMP	6	8
11q13.3	68889918	69589223.5	AMP	6	8
11q13.4	71627053	74357770	AMP	6	8
10p13-p12.2	17275747.5	22617571	AMP	6	8
3q21.2-q26.1	125683802	162501514	AMP	6	8
3q26.1-q26.31	164035254	174942968.5	AMP	6	8

Table 4.2, continued

Cytoband	Median start position	Median end position	CNAs (AMP/DEL)	Number of sample	Percentage % (n=75)
1q22.2	102112312	102885548	AMP	4	5.33
1p36.33	937332	941105	AMP	4	5.33
3p21.31	47076499	49558487	DEL	7	9.3
3p26.3-p26.1	64052	5256910	DEL	7	9.3
8p23.2	3680600	3841195	DEL	6	8

Abbreviation Amp = Amplification; Del = Deletion

Table 4.3: Association of chromosome 7p, 8q, 9p, 11q, 8p and the combination of chromosome 7p, 8q, 9p, 11q with clinico-pathological parameters in OSCC.

Variables	Category	No. of patients (%)	7p			8q			11q			9p			(chr 7p+8q+9p+11q)			8p		
			No change	Gain	P value	No change	Gain	P value	No change	Gain	P value	No change	Gain	P value	No marker	≥1 marker	P value	No change	Loss	P value
Total		75	40	35		46	29		59	16		64	11		25	50		26	49	
Gender	**Male**	26 (34.7)	17 (65.4)	9 (34.6)	0.128	14 (53.8)	12 (46.2)	0.332	18 (69.2)	8 (30.8)	0.146	22 (84.6)	4 (15.4)	1.000	9 (34.6)	17 (65.4)	0.864	15 (57.7)	11 (42.3)	0.072
	Female	49 (65.3)	23 (46.9)	26 (53.1)		32 (65.3)	17 (34.7)		41 (83.7)	8 (16.3)		42 (85.7)	7 (14.3)		16 (32.7)	33 (67.3)		38 (77.6)	11 (22.4)	
Age (years)	**< 45**	12 (16.0)	5 (41.7)	7 (58.3)	0.377	6 (50.0)	6 (50.0)	0.519	8 (66.7)	4 (33.3)	0.271	9 (75.0)	3 (25.0)	0.368	3 (25.0)	9 (75.0)	0.74	8 (66.7)	4(33.3)	0.739
	≥ 45	63 (84.0)	35 (55.6)	28 (44.4)		40 (63.5)	23 (36.5)		51 (81.0)	12 (19.0)		55 (87.3)	8 (12.7)		22 (34.9)	41 (65.1)		45 (71.4)	18 (28.6)	
Smoking	**No**	52 (69.3)	27 (51.9)	25 (48.1)	0.713	33 (63.5)	19 (36.5)	0.569	41 (78.8)	11 (21.2)	1.000	41 (78.8)	11 (21.2)	**0.015***	17 (32.7)	35 (67.3)	0.859	38 (73.1)	14 (26.9)	0.491
	Yes	23 (30.7)	13 (56.5)	10 (43.5)		13 (56.5)	10 (43.5)		18 (78.3)	5 (21.7)		23 (100.0)	0 (0.0)		8 (34.8)	15 (65.2)		15 (65.2)	8 (34.8)	
Drinking	**No**	64 (85.3)	35 (54.7)	29 (45.3)	0.571	37 (57.8)	27 (42.2)	0.186	51 (79.7)	13 (20.3)	0.692	53 (82.8)	11 (17.2)	0.351	20 (31.3)	44 (68.8)	0.49	45 (70.3)	19 (29.7)	1.000
	Yes	11 (14.7)	5 (45.5)	6 (54.5)		9 (81.8)	2 (18.2)		8 (72.7)	3 (27.3)		11 (100.0)	0 (0.0)		5 (45.5)	6 (54.5)		8 (72.7)	3 (27.3)	
Betel quid chewing	**No**	40 (53.3)	21 (52.5)	19 (47.5)	0.877	23 (57.5)	17 (42.5)	0.466	31 (77.5)	9 (22.5)	0.792	34 (85.0)	6 (15.0)	0.93	11 (27.5)	29 (72.5)	0.252	27 (67.5)	13 (32.5)	0.520
	Yes	35 (46.7)	19 (54.3)	16 (45.7)		23 (65.7)	12 (34.3)		28 (80.0)	7 (20.0)		30 (85.7)	5 (14.3)		14 (40.0)	21 (60.0)		26 (74.3)	9 (25.7)	

Table 4.3, continued

Variables	Category	No. of patients (%)	7p			8q			11q			9p			(chr 7p+8q+9p+11q)			8p		
			No change	Gain	P value	No change	Gain	P value	No change	Gain	P value	No change	Gain	P value	No marker	≥1 marker	P value	No change	Loss	P value
Total		75	40	35		46	29		59	16		64	11		25	50		26	49	
Tumour site	**Tongue**	24 (32.0)	14 (58.3)	10 41.7）	0.552	12 (50.0)	12 (50.0)	0.167	20 (83.3)	4 (16.7)	0.499	22 (91.7)	2 (8.3)	0.486	6 (25.0）	18 （75.0）	0.294	18 (75.0)	6 (25.0)	0.572
	Non-tongue**	51 (68.0)	26 (51.0）	25 (49.0)		34 (66.7)	17 (33.3)		39 (76.5)	12 (23.5)		42 (82.4)	9 (17.6)		19 （37.3）	32 （62.7）		35 (68.6)	16 (31.4)	
Tumour size	**T1-T2**	45 (60.0)	31 (68.9)	14 (31.1)	**0.001***	32 (71.1)	13 (28.9)	**0.033***	38 (84.4)	7 (15.6)	0.135	40 (88.9)	5 (11.1)	0.330	20 （44.4）	25 （55.6）	**0.012***	35 (77.8)	10 (22.2)	0.098
	T3-T4	30 (40.0)	9 (30.0)	21 (70.0)		14 (46.7)	16 (53.3)		21 (70.0)	9 (30.0)		24 (80.0)	6 (20.0)		5 （16.7）	25 （83.3）		18 (60.0)	12 (40.0)	
Lymph node metastasis	**Negative**	38 (50.7)	24 (63.2)	14 (36.8)	0.084	27 (71.1)	11 (28.9)	0.080	33 (86.8)	5 (13.2)	0.080	36 (94.7)	2 (5.3)	**0.020***	18 （47.4）	20 （52.6）	**0.009***	30 (78.9)	8 (21.1)	0.110
	Positive	37 (49.3)	16 (43.2)	21 (56.8)		19 (51.4)	18 (48.6)		26 (70.3)	11 (29.7)		28 (75.7)	9 (24.3)		7 (18.9）	30 （81.1）		23 (62.2)	14 (37.8)	
pTNM Staging	**Early stage**	26 (34.7)	19 (73.1)	7 (26.9)	**0.013***	22 (84.6)	4 (15.4)	**0.003***	24 (92.3)	2 (7.7)	**0.036***	25 (96.2)	1 (3.8)	0.085	15 （57.7）	11 （42.3）	**0.001***	23 (88.5)	3 (11.5)	**0.014***
	Advanced stage	49 (65.3)	21 (42.9)	58 (57.1)		24 (49.0)	25 (51.1)		35 (71.4)	14 (28.6)		39 (79.6)	10 (20.4)		10 （20.4）	39 （79.6）		30 (61.2)	19 (38.8)	

*Significant p - value were highlighted in bold and asterisked
**Buccal mucosa, gingiva, lip, floor of mouth, palate

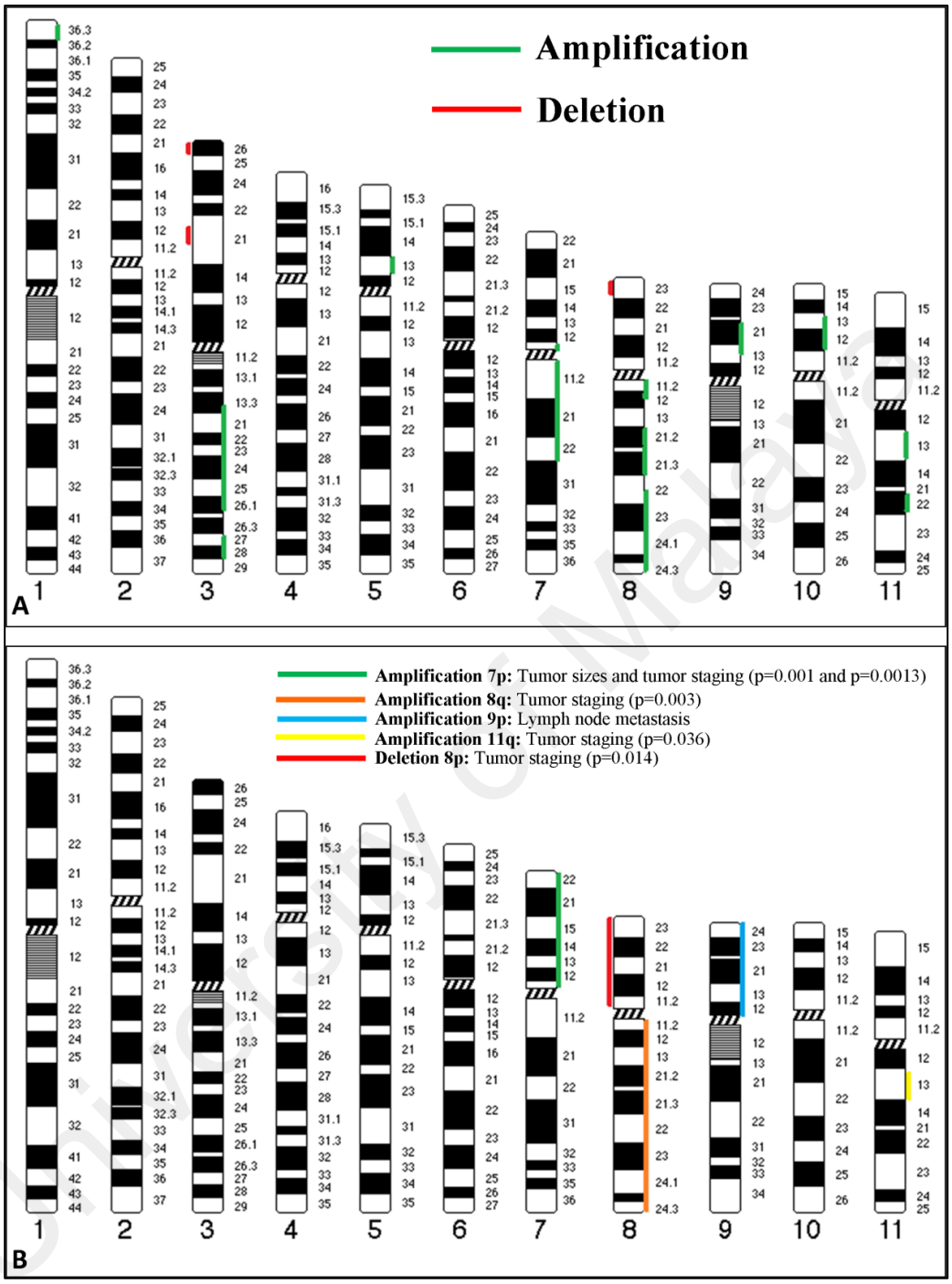


Figure 4.1: The ideogram of amplifications and deletions (A) and the association with clinico-pathological parameters (B) identified in this study using array CGH.

4.1.2 Association of the copy number alterations with survival

To determine whether the CNAs were associated with clinical outcomes, Kaplan-Meier survival analysis was conducted. The overall survival curves of chromosome 7p, 8q, 9p and 11q and the combination of chromosome 7p, 8q, 9p and 11q (amplification ≥ 1 marker vs no amplification) were illustrated in Figure 4.2.

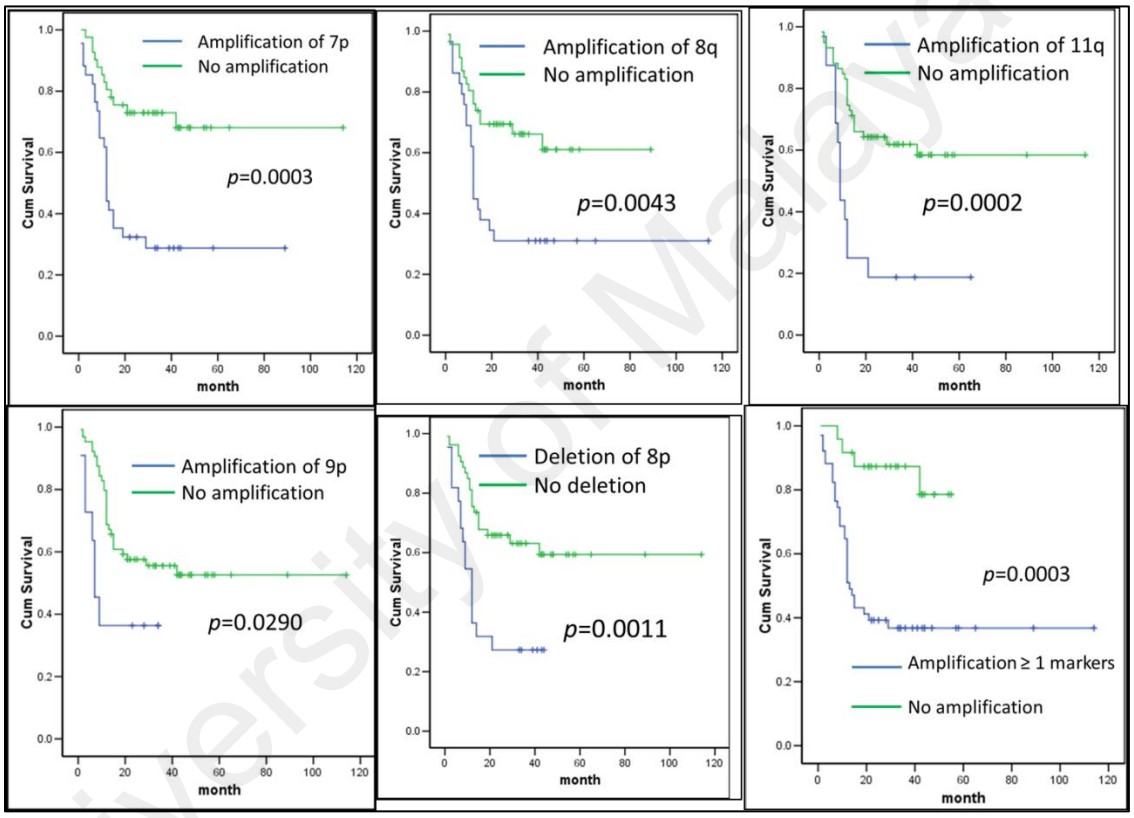


Figure 4.2: Overall survival curves were analyzed according to amplification of chromosome 7p, 8q, 9p, 11q and deletion of chromosome 8p and the combination of chromosome 7p, 8q, 9p, 11q using Kaplan-Meier estimate with log-rank test.

The 3-year survival rates for the amplification and non-amplification of chromosome 8q were 34.48% and 66.13%, respectively. Univariate analysis showed a significant association between amplification of chromosome 8q and poor prognosis ($p < 0.050$). However, the multivariate Cox regression model analysis revealed that amplification of chromosome 8q was not significantly associated with poor prognosis (HRR = 1.677, 95% C1 0.807-3.485, $p = 0.166$) after adjustment for selected socio-demographic (age, gender and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.4), thus, chromosome 8q cannot be considered as an independent prognostic marker.

The 3-year survival rates for the amplification and non-amplification of chromosome 11q were 25.0% and 61.86%, respectively. Univariate ($p < 0.050$) and multivariate Cox regression model analysis revealed that amplification of chromosome 11q was significantly associated with poor prognosis (HRR = 3.211, 95% C1 1.417-7.275, $p = 0.005$) after adjustment for selected socio-demographic (age, gender and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.5), and thus, chromosome 11q remained as an independent prognostic marker.

The 3-year survival rates for the amplification and non-amplification of chromosome 9p were 45.45% and 55.52%, respectively. Similar to chromosome 8q, the amplification of chromosome 9p was significantly associated with poor prognosis in univariate analysis ($p < 0.050$) while the multivariate Cox regression model analysis revealed that amplification of chromosome 9p was not significantly associated with poor prognosis (HRR = 1.378, 95% C1 0.528-3.596, $p = 0.512$) after adjustment for selected socio-demographic (age, gender

and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.6). Thus, chromosome 9p cannot be considered as an independent prognostic marker.

The 3-year survival rates for the amplification and non-amplification of chromosome 7p were 32.35% and 72.93%, respectively. Similar to chromosome 8q and 9p, the amplification of chromosome 7p was significantly related to poor prognosis in univariate analysis ($p < 0.050$). However, the multivariate Cox regression model analysis revealed that amplification of chromosome 7p was not significantly associated with poor prognosis (HRR = 2.022, 95% C1 0.904-4.524, $p = 0.087$) after adjustment for selected socio-demographic (age, gender, and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.7). Therefore, amplification of chromosome 7p is not an independent prognostic marker.

The 3-year survival rates for the deletion and non-deletion of chromosome 8p were 31.82% and 63.10%, respectively. Both univariate analysis ($p < 0.050$) and multivariate Cox regression model analysis revealed that deletion of chromosome 8p was significantly associated with poor prognosis (HRR = 3.088, 95% C1 1.426-6.689, $p = 0.004$) after adjustment for selected socio-demographic (age, gender and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.8). Deletion of chromosome 8p thus remained as an independent prognostic marker.

The 3-year survival rates for the amplification of 1 or more markers and non-amplification of any marker of chr7p8q9p11q were 36.76% and 78.857%, respectively. Both univariate analysis (p = 0.0002) and multivariate Cox regression model analysis revealed that amplification of chr7p8q9p11q was significantly associated with poor prognosis (HRR = 3.554, 95% C1 1.161-10.886, p = 0.026) after adjustment for selected socio-demographic (age, gender and risk habits) and clinico-pathological parameters (tumour sizes, lymph node status and tumour staging) of OSCC (Table 4.9). Thus, amplification of 1 or more markers of chr7p8q9p11q remained as an independent prognostic marker.

Table 4.4: Multivariate Cox regression model analysis of chromosome 8q in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		75			
chromosome 8q	No amplification	46 (61.3)	1.00†		0.166
	Amplification	29 (38.7)	1.677	0.807-3.485	
Gender	Male	26 (34.7)	1.00†		0.909
	Female	49 (65.3)	1.053	0.436-2.538	
Age (years)	< 45	12 (16.0)	1.00†		0.472
	≥ 45	63 (84.0)	1.473	0.512-4.237	
Smoking	No	52 (69.3)	1.00†		0.511
	Yes	23 (30.7)	0.721	0.272-1.912	
Drinking	No	64 (85.3)	1.00†		0.83
	Yes	11 (14.7)	1.148	0.325-4.052	
Betel quid chewing	No	40 (53.3)	1.00†		0.147
	Yes	35 (46.7)	1.83	0.809-4.141	
Tumour size	T1-T2	45 (60.0)	1.00†		**0.030**
	T3-T4	30 (40.0)	2.767	1.013-6.941	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.200
	Positive	37 (49.3)	1.864	0.719-4.836	
pTNM Staging	Early	26 (34.7)	1.00†		0.253
	Advanced	49 (65.3)	2.467	0.524-11.616	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Table 4.5: Multivariate Cox regression model analysis of chromosome 11q in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		75			
chromosome 11q	No amplification	59 (78.7)	1.00†		**0.005**
	Amplification	16 (21.3)	3.211	1.417-7.275	
Gender	Male	26 (34.7)	1.00†		0.398
	Female	49 (65.3)	1.522	0.574-4.035	
Age (years)	< 45	12 (16.0)	1.00†		0.461
	≥ 45	63 (84.0)	1.471	0.528-4.102	
Smoking	No	52 (69.3)	1.00†		0.623
	Yes	23 (30.7)	0.78	0.289-2.103	
Drinking	No	64 (85.3)	1.00†		0.855
	Yes	11 (14.7)	0.886	0.241-3.257	
Betel quid chewing	No	40 (53.3)	1.00†		0.405
	Yes	35 (46.7)	1.449	0.606-3.466	
Tumour size	T1-T2	45 (60.0)	1.00†		**0.045**
	T3-T4	30 (40.0)	2.504	1.019-6.151	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.313
	Positive	37 (49.3)	1.618	0.635-4.123	
pTNM Staging	Early	26 (34.7)	1.00†		0.145
	Advanced	49 (65.3)	3.121	0.676-14.413	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Table 4.6: Multivariate Cox regression model analysis of chromosome 9p in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		75			
chromosome 9p	No amplification	64 (85.3)	1.00†		0.512
	Amplification	11 (14.7)	1.378	0.528-3.596	
Gender	Male	26 (34.7)	1.00†		0.895
	Female	49 (65.3)	1.063	0.431-2.620	
Age (years)	< 45	12 (16.0)	1.00†		0.568
	≥ 45	63 (84.0)	1.371	0.465-4.042	
Smoking	No	52 (69.3)	1.00†		0.659
	Yes	23 (30.7)	0.793	0.283-2.223	
Drinking	No	64 (85.3)	1.00†		0.926
	Yes	11 (14.7)	1.061	0.302-3.725	
Betel quid chewing	No	40 (53.3)	1.00†		0.161
	Yes	35 (46.7)	1.829	0.787-4.249	
Tumour size	T1-T2	45 (60.0)	1.00†		**0.023**
	T3-T4	30 (40.0)	2.963	1.158-7.578	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.374
	Positive	37 (49.3)	1.568	0.582-4.225	
pTNM Staging	Early	26 (34.7)	1.00†		0.154
	Advanced	49 (65.3)	3.058	0.657-14.220	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Table 4.7: Multivariate Cox regression model analysis of chromosome 7p in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		75			
chromosome 7p	No amplification	40 (53.3)	1.00†		0.087
	Amplification	35 (46.7)	2.022	0.904-4.524	
Gender	Male	26 (34.7)	1.00†		0.761
	Female	49 (65.3)	0.874	0.368-2.077	
Age (years)	< 45	12 (16.0)	1.00†		0.317
	≥ 45	63 (84.0)	1.745	0.586-5.197	
Smoking	No	52 (69.3)	1.00†		0.405
	Yes	23 (30.7)	0.667	0.257-1.732	
Drinking	No	64 (85.3)	1.00†		0.976
	Yes	11 (14.7)	0.981	0.280-3.439	
Betel quid chewing	No	40 (53.3)	1.00†		0.174
	Yes	35 (46.7)	1.788	0.773-4.136	
Tumour size	T1-T2	45 (60.0)	1.00†		0.073
	T3-T4	30 (40.0)	2.379	0.921-6.144	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.269
	Positive	37 (49.3)	1.709	0.661-4.419	
pTNM Staging	Early	26 (34.7)	1.00†		0.187
	Advanced	49 (65.3)	2.774	0.610-12.620	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Table 4.8: Multivariate Cox regression model analysis of chromosome 8p in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		75			
chromosome 8p	No deletion	53 (70.7)	1.00†		**0.004**
	Deletion	22 (29.3)	3.088	1.426-6.689	
Gender	Male	26 (34.7)	1.00†		0.380
	Female	49 (65.3)	1.535	0.589-4.001	
Age (years)	< 45	12 (16.0)	1.00†		0.348
	≥ 45	63 (84.0)	1.666	0.573-4.839	
Smoking	No	52 (69.3)	1.00†		0.298
	Yes	23 (30.7)	0.596	0.224-1.580	
Drinking	No	64 (85.3)	1.00†		0.768
	Yes	11 (14.7)	1.215	0.333-4.430	
Betel quid chewing	No	40 (53.3)	1.00†		0.224
	Yes	35 (46.7)	1.68	0.728-3.875	
Tumour size	T1-T2	45 (60.0)	1.00†		**0.017**
	T3-T4	30 (40.0)	3.04	1.217-7.594	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.182
	Positive	37 (49.3)	1.906	0.739-4.913	
pTNM Staging	Early	26 (34.7)	1.00†		0.209
	Advanced	49 (65.3)	2.67	0.577-12.355	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Table 4.9: Multivariate Cox regression model analysis of the combination of chromosome 7p, 8q, 9p and 11q in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	**95% CI**	**p value**
Total		75			
chr7p8q9p11q	No amplification	24 (32.0)	1.00†		**0.026**
	Amplification ≥ 1 marker	51 (68.0)	3.554	1.161-10.886	
Gender	Male	26 (34.7)	1.00†		0.969
	Female	49 (65.3)	1.017	0.446-2.316	
Age (years)	< 45	12 (16.0)	1.00†		0.432
	≥ 45	63 (84.0)	1.525	0.532-4.370	
Smoking	No	52 (69.3)	1.00†		0.490
	Yes	23 (30.7)	0.719	0.281-1.838	
Drinking	No	64 (85.3)	1.00†		0.668
	Yes	11 (14.7)	1.314	0.377-4.583	
Betel quid chewing	No	40 (53.3)	1.00†		0.074
	Yes	35 (46.7)	2.078	0.931-4.640	
Tumour size	T1-T2	45 (60.0)	1.00†		0.061
	T3-T4	30 (40.0)	2.385	0.960-5.924	
Lymph node metastasis	Negative	38 (50.7)	1.00†		0.319
	Positive	37 (49.3)	1.615	0.630-4.141	
pTNM Staging	Early	26 (34.7)	1.00†		0.214
	Advanced	49 (65.3)	2.652	0.569-12.363	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

4.1.3 TaqMan copy number assay of LRP12, TPM2, EGFR, FSCN1, CCND1, CLPTM1L, CHL1 and CSMD1

Selected CNAs in terms of amplification and deletion regions were confirmed through qPCR validation. Amplifications and deletions of the genomic regions were defined on the basis of differences between patient's copy number and the wild-type copy number (i.e. a copy number around 2). Each CNA associated gene from the amplified and deleted regions were validated using qPCR copy number assay analysis with 66 OSCC samples which overlapped with 75 array CGH samples. The amplification/deletion of the selected genes identified from array CGH and qPCR were illustrated in Figure 4.3. Out of 26 samples that showed amplification of chromosome 8q22.3 (LRP12) in array CGH, 13 (50%) samples showed amplification in the qPCR copy number assay validation. Approximately 50% (7/14), 54.5% (6/11), 59.4%, 71.9% and 75% of the samples that showed amplification in array CGH analysis for CCND1 (chromosome 11q13.3), TPM2 (chromosome 9p13.3), FSCN, EGFR (chromosome 7p11.2) and CLPTM1L (chromosome 5p15.33) respectively were validated in qPCR copy number analysis. As for the deletion CNAs, approximately 33.3% (7/21) and 36.8% (7/19) of the samples that showed deletions in array CGH analysis for CSMD1 (chromosome 8p23.2) and CHL1 (chromosome 3p26.3) respectively were validated in qPCR copy number analysis (Figure 4.3).

In chi-square analysis, the results showed that LRP12, TPM2, EGFR, FSCN1, CCND1, CLPTM1L, CHL1 and CSMD1 were not associated with socio-demographic and clinico-pathologic parameters. Additionally, Kaplan-Meier survival analysis also showed that LRP12, TPM2, FSCN1, CCND1, CLPTM1L, CHL1 and CSMD1 were not associated with poor prognosis. Only amplification of EGFR showed a trend towards association with poor prognosis ($p = 0.060$). In order to validate the combination of four

chromosomes (chr7p8q9p11q) that was reported previously, the candidate genes that harboured within this combination of 4 amplified chromosomes (chr7p8q9p11q) namely EGFR (7p), LRP12 (8q), TPM2 (9p) and CCND1 (11q) were subjected to statistical analysis. In addition, this study also tested whether the combination of the four genetic markers (CCND1, EGFR, TPM2 and LRP12) could further increase the sensitivity and specificity as a prognostic marker in OSCC samples. The combined AUC for all the markers was 0.621 which is higher than the single markers of the genetic markers. The OSCC patients were divided into those who have a cumulative score of 0 markers (group 1) versus those with a cumulative score of 1, 2, 3, or 4 markers (group 2). The Kaplan-Meier survival curves showed a significant survival probability difference between patients in groups 1 and 2 ($p = 0.045$) (Figure 4.4). Furthermore, the Cox regression multivariate model revealed that the 4 combined genetic marker remained as an independent prognostic factor with hazard risk ratio (HRR = 2.34) towards death in patients with amplification of 1 or more markers after adjustment for age, gender, risk habits and clinico-pathologic parameters (tumour sizes, lymph node metastasis, tumour staging and tumour differentiation) (Table 4.10).

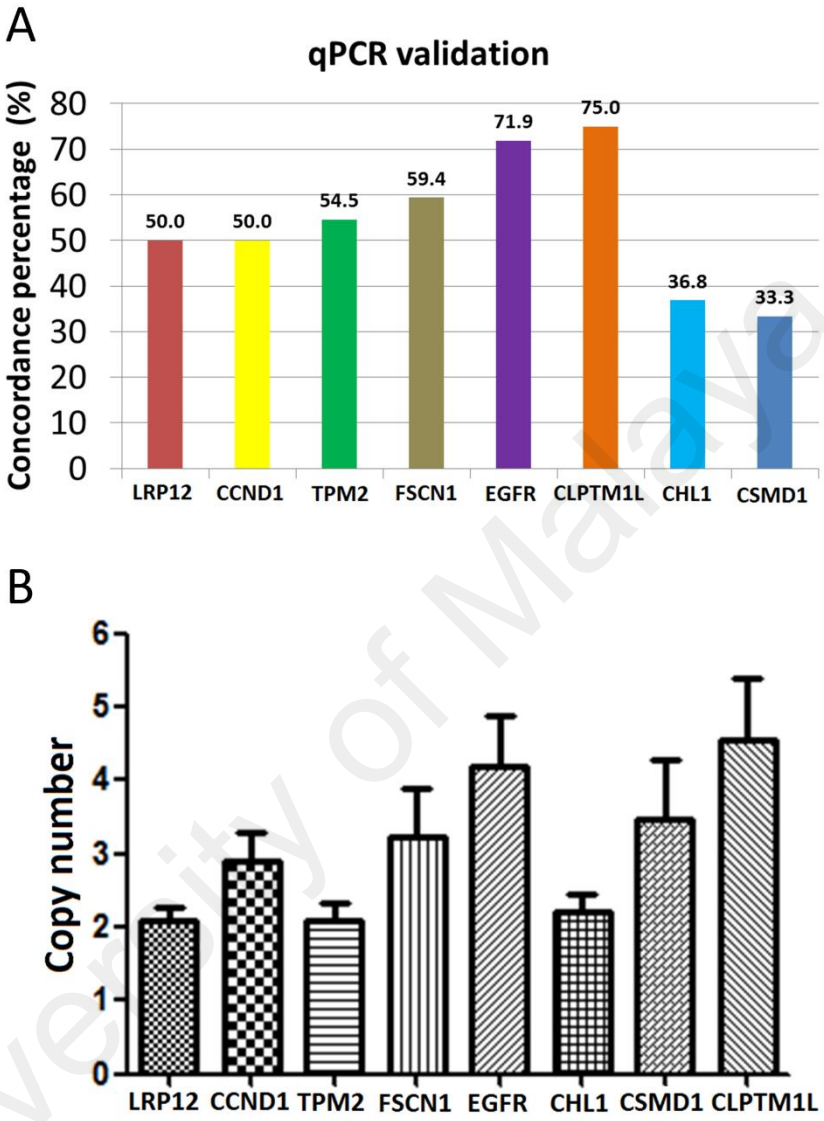


Figure 4.3: (A) Concordance percentage for amplification of LRP12 (chr 8q), CCND1 (chr 11q), TPM2 (chr 9p), FSCN1 (chr 7p), EGFR (chr 7p), CLPTM1L (chr 5p) and deletion of CHL1 (chr 3p) and CSMD1 (chr 8p) identified using array CGH and validated using qPCR copy number analysis in 66 OSCC samples. (B) Results for the copy number of LRP12 (chr 8q), CCND1 (chr 11q), TPM2 (chr 9p), FSCN1 (chr 7p), EGFR (chr 7p), CLPTM1L (chr 5p), CHL1 (chr 3p) and CSMD1 (chr 8p) was performed using qPCR. A copy number of 2 were considered to be diploid, a copy number with < 2 was indicative of deletion, whereas a copy number of > 2 was held to indicate amplification. The error bars represent the standard error among three replicates.

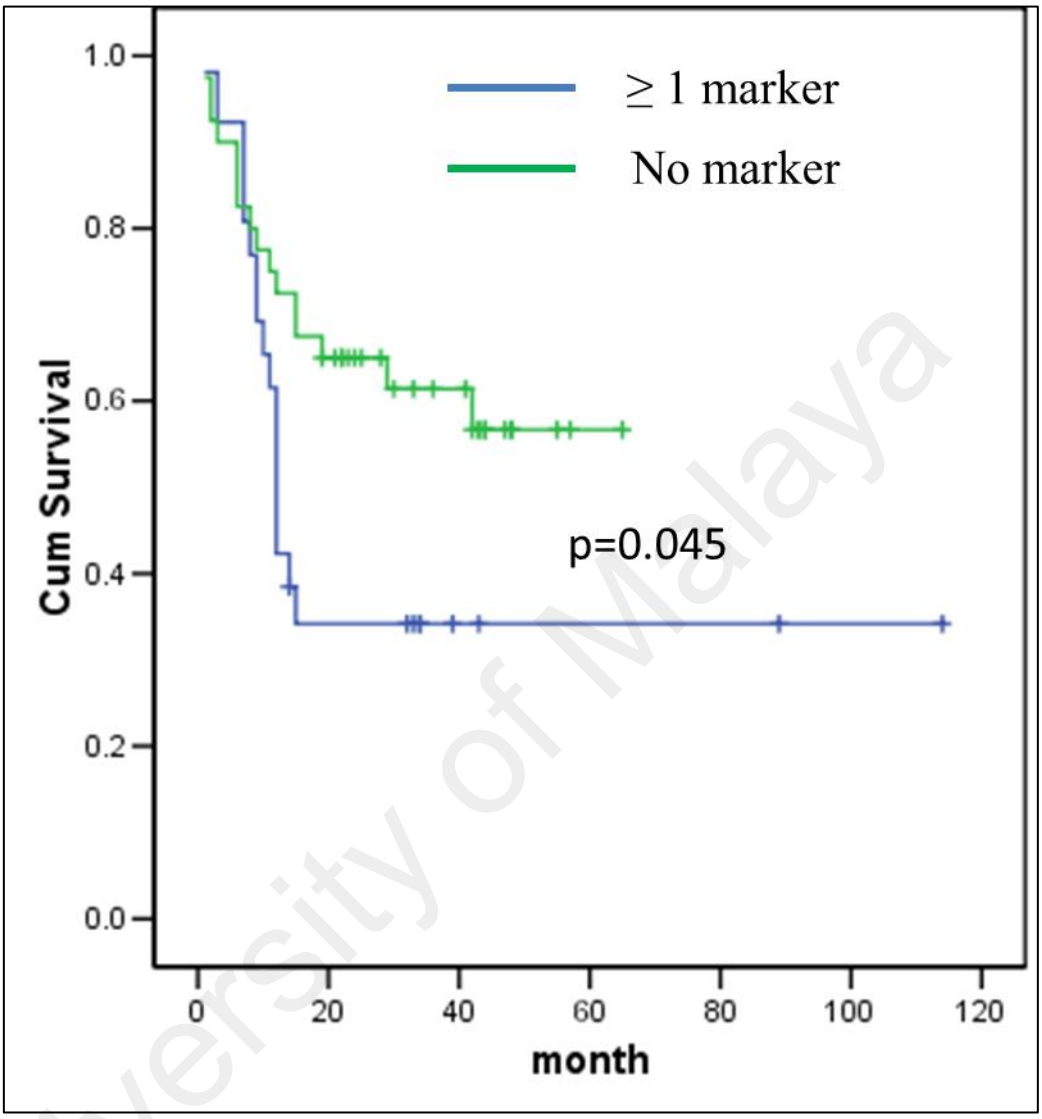


Figure 4.4: Overall survival curves were analyzed according to the 4 combined genetic marker (EGFR+CCND1+TPM2+LRP12) using Kaplan-Meier estimate with log-rank test.

Table 4.10: Multivariate Cox regression model analysis of four combined genetic marker consisting of EGFR, TPM2, CCND1 and LRP12 in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
Total		66	**HRR**	**95% CI**	**p value**
Four combined genetic marker	no marker	40 (60.6)	1.00†		**0.038**
	≥ 1 marker	26 (39.4)	2.343	1.047-5.244	
Gender	Male	24 (36.4)	1.00†		0.508
	Female	42 (63.6)	1.4	0.517-3.794	
Age (years)	< 45	12 (18.2)	1.00†		0.977
	≥ 45	54 (81.8)	1.016	0.331-3.119	
Smoking	No	45 (68.2)	1.00†		0.589
	Yes	21 (31.8)	0.744	0.254-2.178	
Drinking	No	57 (86.4)	1.00†		0.398
	Yes	9 (13.6)	1.899	0.429-8.406	
Betel quid chewing	No	35 (53.0)	1.00†		0.107
	Yes	31 (47.0)	2.089	0.852-5.122	
Tumour size	T1-T2	37 (56.1)	1.00†		**0.018**
	T3-T4	29 (43.9)	3.36	1.23-9.177	
Lymph node metastasis	Negative	33 (50.0)	1.00†		0.228
	Positive	33 (50.0)	1.843	0.682-4.983	
pTNM Staging	Early	22 (33.3)	1.00†		0.322
	Advanced	44 (66.7)	2.27	0.449-11.484	
Differentiation	Well	27 (40.9)	1.00†		0.299
	Moderate and poor	39 (59.1)	1.526	0.687-3.388	

CI: confidence interval
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sizes, lymph node metastasis and pathological tumour staging]

Objective 2: To identify the pathways and networks implicated in OSCC pathogenesis.

4.2 Biological process analysis

This study aimed to present the results in accordance with the specific objectives as such the first objective is to determine and validate the CNAs of OSCC using array CGH and qPCR copy number assay.

To explore the potential effects of the chromosomal alterations implicated in the molecular mechanism of oral tumourigenesis, the biological functions annotations and biological network that were associated with CNAs were further analyzed. The top significant signalling pathways were identified as integrin-linked kinase signalling pathway (Table 4.11). The molecular and cellular functions were categorized into five different groups including cell death and survival, cellular function and maintenance, cellular development, cellular growth and proliferation, and cellular movement. The associated top five molecular and cellular functions of these categories were listed in Table 4.12. The top molecular and cellular functions were reported as cell death and survival, cellular function and maintenance which is mostly associated with colony survival of cells (p = 8.72E-05) through contributing of ATR, CA9, CCND1, FANCG, RAD21, RB1CC1, TERC and TNFSF10 genes. The category of diseases was reported as related to head and neck squamous cell cancer with 26 genes associated with this cancer. These 26 genes were MALAT1, MRC1, POLQ, CCND1, SOX17, LIFR, FGF4, mir-15, FGF3, SHANK2, RAD21, EGFR, FGF19, PPFIA1, TPCN2, MECOM, ANO1, ORAOV1, FADD, DDX58, EPPK1, LYN, ATR, SETD2, MYEOV and CTTN.

Table 4.11: Top significant pathways that associated with CNAs associated genes

Ingenuity Canonical Pathways	-log (p-value)	Molecules
ILK Signaling	3.26E00	RELA,SNAI2,CFL1,ACTB,PPP2R5B,ACTN3,VEGFB,VIM,PIK3R4,RICTOR,PPP1R14B,CCND1,PPP2R3A,RHOD,RHOA,PPM1L,RPS6KA4,PIK3CB,GSK3B,TESK1,ITGB5,MYL3
mTOR Signaling	3.23E00	EIF3H,PPP2R5B,RAC1,VEGFB,EIF3E,PIK3R4,RICTOR,PLD1,FAU,PRKCI,RPS20,EIF3B,PPP2R3A,RHOD,RHOA,PPM1L,PRKAA1,MRAS,RPS6KB2,PIK3CB,RPS6KA4,RPS3
Tight Junction Signaling	2.59E00	RELA,CLDN11,ACTB,HSF1,PPP2R5B,CLDN18,MARK2,CPSF1,RAC1,PRKAR2A,MYLK,GPAA1,PRKCI,PPP2R3A,CLDN1,RHOA,PPM1L,PRKAR1B,MYL3
Role of CHK Proteins in Cell Cycle Checkpoint Control	2.47E00	PPP2R3A,RAD9A,PPP2R5B,PPM1L,E2F5,ATR,NBN,RAD1,CDC25A
UVA-Induced MAPK Signaling	2.47E00	TIPARP,PARP15,PARP10,RPS6KB2,MRAS,PLCB3,PIK3CB,RPS6KA4,PIK3R4,PARP9,EGFR,PARP14
NRF2-mediated Oxidative Stress Response	2.28E00	DNAJB8,ACTB,DNAJC13,DNAJC1,PIK3R4,MAFK,DNAJA1,DNAJB13,DNAJC21,PRKCI,DNAJC4,STIP1,VCP,MRAS,FOSL1,PIK3CB,GSK3B,GSTP1,DNAJB5
p70S6K Signaling	2.19E00	BAD,PPP2R5B,PIK3R4,PLD1,GNAI2,PRKCI,PPP2R3A,PPM1L,MRAS,LYN,PLCB3,PIK3CB,AGTR1,EGFR
HIPPO signaling	2.1E00	YAP1,PPP2R3A,WWTR1,PPP2R5B,PPM1L,SCRIB,MST1,PPP1CA,PPP1R14B,RASSF1,SKP2

Table 4.11, continued

Ingenuity Canonical Pathways	-log(p-value)	Molecules
p53 Signaling	2.05E00	PRKDC,SNAI2,TP63,TOPBP1,STAG1,ADGRB1,ATR, PIK3CB,GSK3B,PIK3R4,CCND1,GML
Death Receptor Signaling	1.88E00	FADD,RELA,TIPARP,PARP15,PARP10,ACTB,TNFSF 10,PARP9,BIRC3,BIRC2,PARP14

Table 4.12: Top significant molecular and cellular functions associated with CNAs associated genes.

Molecular and Cellular Functions	Function annotation	p values	Molecule Genes
Cell Death and Survival	colony survival of cells	1.89E-04	ATR,CA9,CCND1,FANCG,RAD21,RB1CC1,TERC,TNFSF10
	colony survival of tumour cell lines	9.17E-04	CA9,CCND1,FANCG,RAD21,RB1CC1,TERC,TNFSF10
	cell viability of fibroblast cell lines	1.07E-03	ATR,CEBPD,FANCG,MUS81,NBN,RAD9A
	apoptosis of kidney cell lines	1.67E-03	ADRBK1,BAD,BCL6,BIRC2,BIRC3,CALB1,CASR,CHMP5,FADD,FGF19,GSK3B,ITPR1,LYN,PRKAA1,RAC1,RAD21,RNF13,RNF216,SYVN1,TNFSF10,TRPS1,UNC13B,VOPP1,YAP1
	cell death of kidney cell lines	2.95E-03	ADRBK1,BAD,BCL6,BIRC2,BIRC3,CALB1,CASR,CHMP5,FADD,FGF19,GSK3B,GSTP1,IP6K2,ITPR1,LYN,NMNAT3,PRKAA1,PRKDC,RAC1,RAD21,RASSF1,RNF13,RNF216,SYVN1,TNFSF10,TRPS1,UNC13B,VOPP1,YAP1
Cellular Function and Maintenance	colony survival of cells	1.89E-04	ATR,CA9,CCND1,FANCG,RAD21,RB1CC1,TERC,TNFSF10
	colony survival of tumour cell lines	9.17E-04	CA9,CCND1,FANCG,RAD21,RB1CC1,TERC,TNFSF10
	autophagy of epithelial cells	3.16E-03	FADD,TNFSF10
	colony survival of breast cancer cell lines	3.16E-03	CA9,RB1CC1
	uptake of bacteria	5.24E-03	OTUB1,RAC1,RHOA

Table 4.12, continued

Molecular and Cellular Functions	Function annotation	p values	Molecule Genes
Cellular Development	proliferation of lung cancer cell lines	4.46E-04	ALDH1L1,BAD,BOP1,CAMP,CCND1,CDCA5,COPB2,ECT2,EGFR,FOSL1,GOLPH3,ITPR1,mir-15,mir-191,mir-192,mir-194,MMP1,MST1R,PIK3CB,PLS1,POLD4,PRKCI,RAC1,RASSF1,RELA,SEMA3B,SKP2,TNFSF10,TP63,TRPC1,TUSC2,YAP1
	proliferation of breast cell lines	2.24E-03	CCND1,EGFR,EIF3E,NUDT1,PGR,PRLR,RAC1,RELA,RHOA,RICTOR,TP63,TRAIP,WWTR1,YAP1
	maturation of mononuclear leukocytes	3.16E-03	NCK1,TNFSF10
	epithelial-mesenchymal transition of bladder cancer cell lines	9.14E-03	MALAT1,TP63
	proliferation of keratinocytes	9.55E-03	CASR,EGFR,SNAI2,TERC,TP63,TRH,YAP1
Cellular Growth and Proliferation	proliferation of lung cancer cell lines	4.46E-04	ALDH1L1,BAD,BOP1,CAMP,CCND1,CDCA5,COPB2,ECT2,EGFR,FOSL1,GOLPH3,ITPR1,mir-15,mir-191,mir-192,mir-194,MMP1,MST1R,PIK3CB,PLS1,POLD4,PRKCI,RAC1,RASSF1,RELA,SEMA3B,SKP2,TNFSF10,TP63,TRPC1,TUSC2,YAP1
	proliferation of breast cell lines	2.24E-03	CCND1,EGFR,EIF3E,NUDT1,PGR,PRLR,RAC1,RELA,RHOA,RICTOR,TP63,TRAIP,WWTR1,YAP1
	proliferation of epithelial cells	9.46E-03	CASR,CCND1,EGFR,GOLPH3,MST1R,SKP2,SNAI2,TERC,TP63,TRH,YAP1
	proliferation of keratinocytes	9.55E-03	CASR,EGFR,SNAI2,TERC,TP63,TRH,YAP1
	colony formation of bone cancer cell lines	1.32E-02	DROSHA,MAD1L1,mir-192,YAP1

Table 4.12, continued

Molecular and Cellular Functions	Function annotation	p values	Molecule Genes
Cellular Movement	migration of carcinoma cell lines	6.85E-04	BRMS1,CAMP,CTTN,DDX58,EGFR,LYN,MEN1,mir-15,mir-612,MMP1,MMP3,MMP7,PRKCI,RAC1,RASSF1,RHOA,SNAI2,TP63,TRH,VIM,VPS28
	migration of tumour cell lines	1.65E-03	ARRB1,BAG1,BRMS1,CA9,CAMP,CCL19,CCND1,CST6,CTTN,DAB2,DDX58,DROSHA,E2F5,ECT2,EGFR,ESRRA,FOSL1,FSCN1,GDNF,GNA12,GOLPH3,GPER1,GRINA,GSK3B,IP6K2,ITGB5,ITPR1,LYN,MALAT1,MEN1,mir-15,mir-326,mir-612,MME,MMP1,MMP3,MMP7,MRAS,MST1,MST1R,NDUFAF3,P2RY2,PDGFA,PGR,PLD1,PLXNA1,PLXNB1,PPFIA1,PRKCI,PTGER4,PTP4A3,RAC1,RAD9A,RARRES1,RASSF1,RELA,RHOA,RICTOR,RUVBL1,SDCBP,SNAI2,SST,TNFSF10,TP63,TRH,VCP,VEGFB,VIM,VPS28,WWTR1
	cell movement of lung cancer cell lines	2.36E-03	BRMS1,CAMP,EGFR,FOSL1,MEN1,MMP1,MMP7,PRKCI,RAC1,RASSF1,RHOA,TP63,TRH,VIM,VPS28
	cell movement of carcinoma cell lines	2.36E-03	BRMS1,CAMP,CTTN,DDX58,EGFR,FOSL1,LY6D,LYN,MEN1,mir-15,mir-612,MMP1,MMP3,MMP7,PRKCI,RAC1,RASSF1,RHOA,SNAI2,TP63,TRH,VIM,VPS28
	migration of lung cancer cell lines	2.52E-03	BRMS1,CAMP,EGFR,MEN1,MMP1,MMP7,PRKCI,RAC1,RASSF1,RHOA,TRH,VIM,VPS28

Results of network analysis of 1427 CNA associated genes have been summarized in Table 4.13. The most significant network was linked to cell death and survival, cellular movement, cellular development. Top significant network harbours 73 genes and among which the major cores CCND1, RELA, TP63 and EGFR were hub nodes in the network, and formed interconnected auto-regulatory and feed forward circuitry (Figure 4.5). The associated network functions analysis revealed that the core-bound genes are mostly relevant to the growth of tumour proliferation, evade apoptosis signal to promote cell survival and metastasis. Apart from that, this analysis also revealed putative novel oncogenes, namely ISG15 and MMP13 involved in the network signalling that linked with cell death and survival, cellular movement, cellular development. These genes were prioritized for further evaluation and characterization in OSCC by quantification of mRNA and protein expression levels as their clinical impact in oral cancer is poorly understood and literature suggests a high probability of involvement in tumourigenesis. These genes were found to harbor in rare amplicons (chromosome 1p36.33 and 11q22.2), however, it has been hypothesized that even rare amplicons would be informative regarding genes crucial in tumor development as the expression may be altered by either copy number changes or other mechanisms by disrupting the upstream or downstream genes in different mechanisms that control a particular pathway (Snijders *et al.*, 2005). Therefore, in order to test this hypothesis whether ISG15 is a driver gene in OSCC (as MMP13 roles in oral tumorigenesis has been studied previously). Therefore, ISG15 was selected for further downstream analysis which is to determine its gene and protein expression in OSCC samples as well as its functional roles via *in vitro* study using OSCC cell line models based on its involvement in the oncogenic network (cell death and survival, cellular movement, cellular development).

Table 4.13: Top significant networks and the associated network functions that linked with CNAs associated genes.

No	Top Diseases and Functions	Score	Focus Molecules	Molecules in Network
1	Cell Death and Survival, Cellular Movement, Cellular Development	73	87	14-3-3,26s Proteasome,ADRBK1,AIP,ANGPT1,ARRB1,ATR,Actin,Akt,Ap1,BAD,BAG1,BCR(complex),BIRC2,BIRC3,CAMP,CARD11,CARD6,CASR,CCND1,CD3,CD86,CDC25A,CEBPD,CORO1B,CPNE4,CPT1A,CYP11B2,Caspase3/7,Cdk,Creb,CyclinA,CyclinE,DAB2,DDX58,EGFR,ERK,ERK1/2,FActin,FADD,FOSL1,FSCN1,Focaladhesionkinase,GDNF,GPER1,GSK3B,Gsk3,Hdac,Histoneh3,Histoneh4,Hsp27,Hsp70,Hsp90,IFNBeta,IKK(complex),ISG15,IgG,Interferonalpha,Jnk,KAT5,LY6E,LY6K,LYN,MAP2K1/2,MAP3K11,MAP4K2,MBD4,MMP1,MMP13,MST1R,MTORC1,MTORC2,MUS81,MYLK,Mek,Mmp,NEU3,NFkB(complex),Nfat(family),OVOL1,P2RY2,P2RY6,P38MAPK,PARP,PDGFBB,PELI3,PGR,PI3K(complex),PI3K(family),PIK3CB,PLD1,PLSCR1,PRKCI,PRKDC,PRLR,PTGER4,PTP4A3,Pkc(s),Pld,RAC1,RASSF1,RELA,RHOA,RICTOR,RIPK2,RNF216,RPS3,RUSC2,Rac,Ras,Rashomolog,Rock,SCRIB,SDCBP,SEMA3B,SHARPIN,SKIL,SKP2,SNAI2,Shc,Smad2/3,TCR,TNFSF10,TP63,TRAIP,TRPC1,TRPC6,UBA7,USP19,VCP,VEGFB,VIM,VOPP1,Vegf,WWTR1,YAP1,caspase,estrogenreceptor,mir-506,p85 (pik3r)

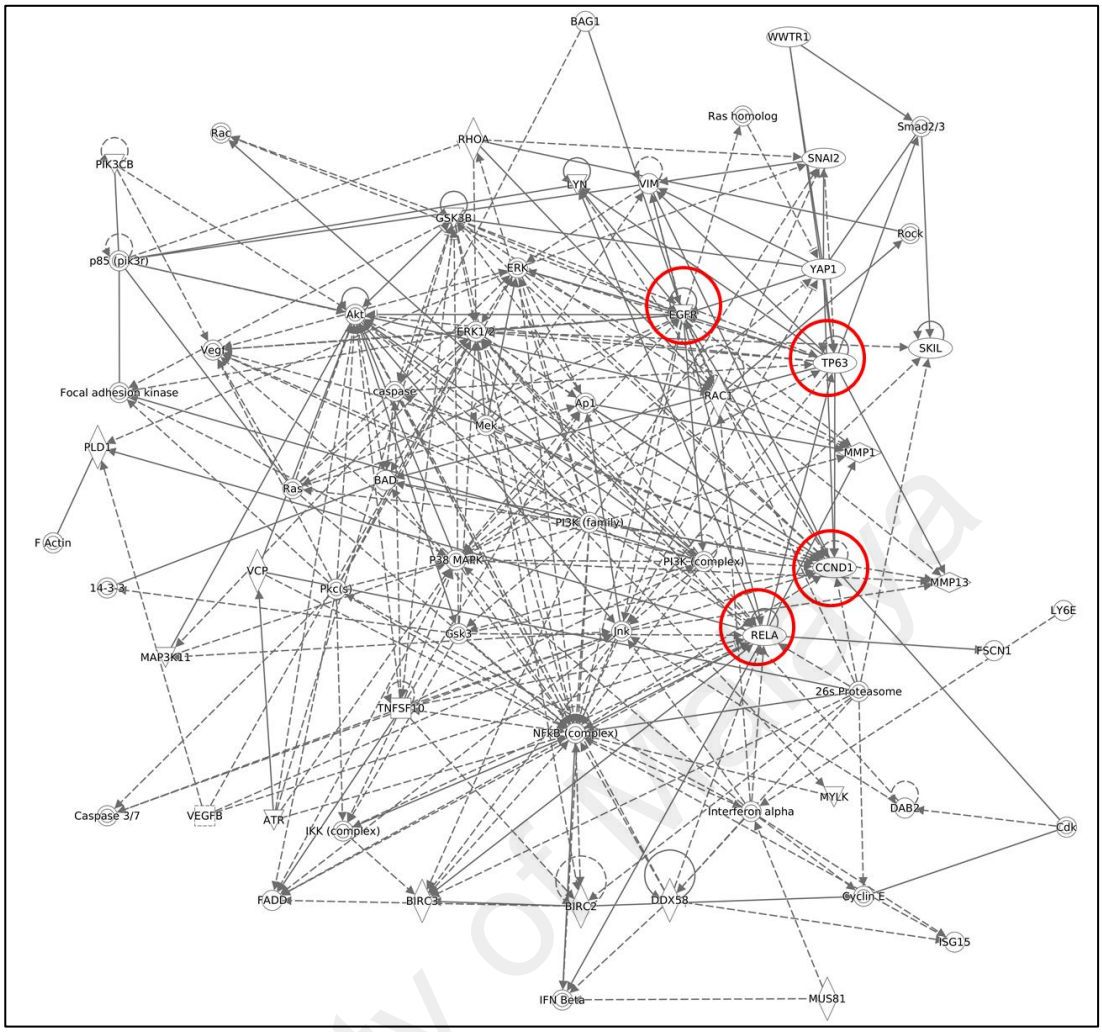


Figure 4.5: The top significant network was related to cell death and survival, cellular movement, cellular development. Top significant network harbours 73 genes among which the major cores CCND1, RELA, TP63 and EGFR (located in the red circle) were hub nodes in the network, and formed interconnected auto-regulatory and feed forward circuitry.

Objective 3: To identify and determine the mRNA and protein expression of candidate genes and their correlation with clinical and socio-demographic parameters as well as the survival of patients.

4.3 Copy number, mRNA and protein expression of candidate genes in OSCC

4.3.1 MMP13

4.3.1.1 MMP13 gene copy number, mRNA and protein expression in OSCC

Amplification of MMP13 was identified in 5.33% of the OSCC samples (4 out of 75) in chromosome 11q22.2 using array CGH (Table 4.2). In line with this, the MMP13 mRNA was found to be expressed significantly at a high level in 95.59% of the OSCC samples (65 out of 68) with an average gene expression fold change of 276.28 compared to the non-cancer oral mucosa samples with fold change of 1 ($p = 0.002$) (Figure 4.6). In the current study, protein expression of MMP13 was examined by IHC analysis. Ovarian cancer tissue was used as the positive control for immunohistochemistry of MMP13 protein expression. The positive control tissue demonstrated strong staining in the cytoplasm of tumour cells (Figure 4.7). Moreover, the negative control (OSCC tissue) for immunohistochemistry showed an absence of cytoplasmic and nuclear staining (Figure 4.7). MMP13 was expressed in all of the OSCC and OED samples. IHC analysis clearly demonstrated that MMP13 protein expression was specifically detected in OED and OSCC indicating that this gene is likely to be associated with both OED and OSCC. In IHC analysis of MMP13 protein, the epithelial cells of non-cancer oral mucosal tissues showed a negative staining. Weak to moderate staining was seen in the cytoplasm of epithelial cells of the basal and spinous layers in OED. More than 75% of OSCCs displayed strong staining in the cytoplasm of epithelial tumour cells. All the non-cancer, OED and OSCC tissue samples demonstrated moderate MMP13 immunostaining of the stromal compartment

including the inflammatory cells. There was no MMP13 protein expression was found innon-cancer oral mucosal tissues whereas MMP13 was over-expressed in OED and OSCC, respectively. A representative haematoxylin and eosin (H&E) and IHC stained image for protein expression group of MMP13 is shown in Figure 4.7.

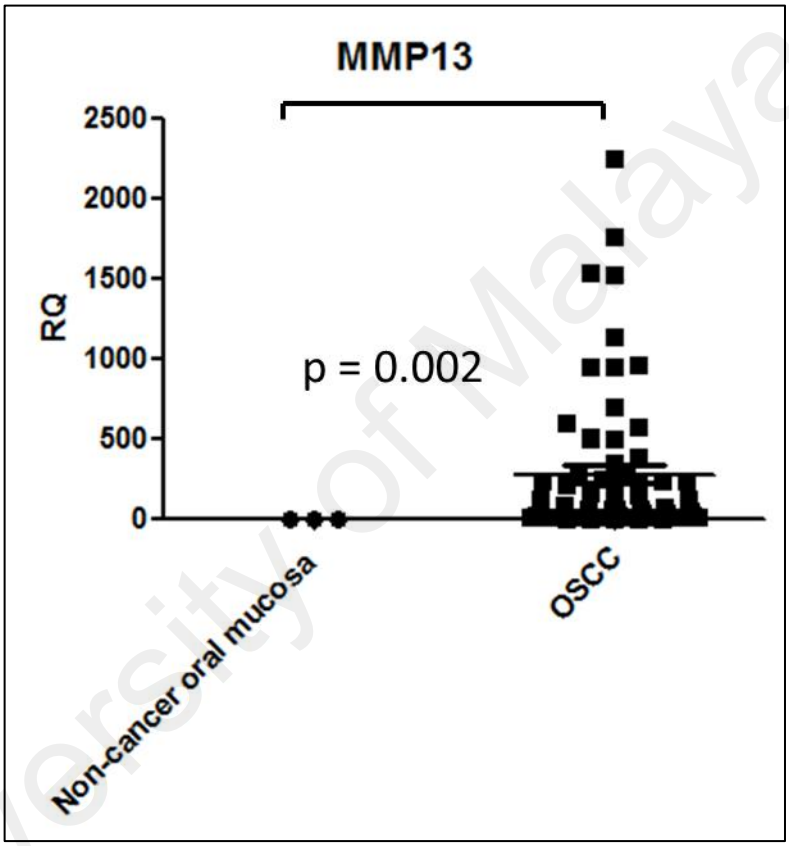


Figure 4.6: Average gene expression fold change of MMP13 in OSCC (n = 68) compared to normal oral mucosa sample (n = 5). Gene expression fold change of MMP13 was 276.28 while the gene expression between OSCC and non-cancer oral mucosa was statistically different (p < 0.005). The RQ for non-cancer oral mucosa of MMP13 was 1 and used for normalization.

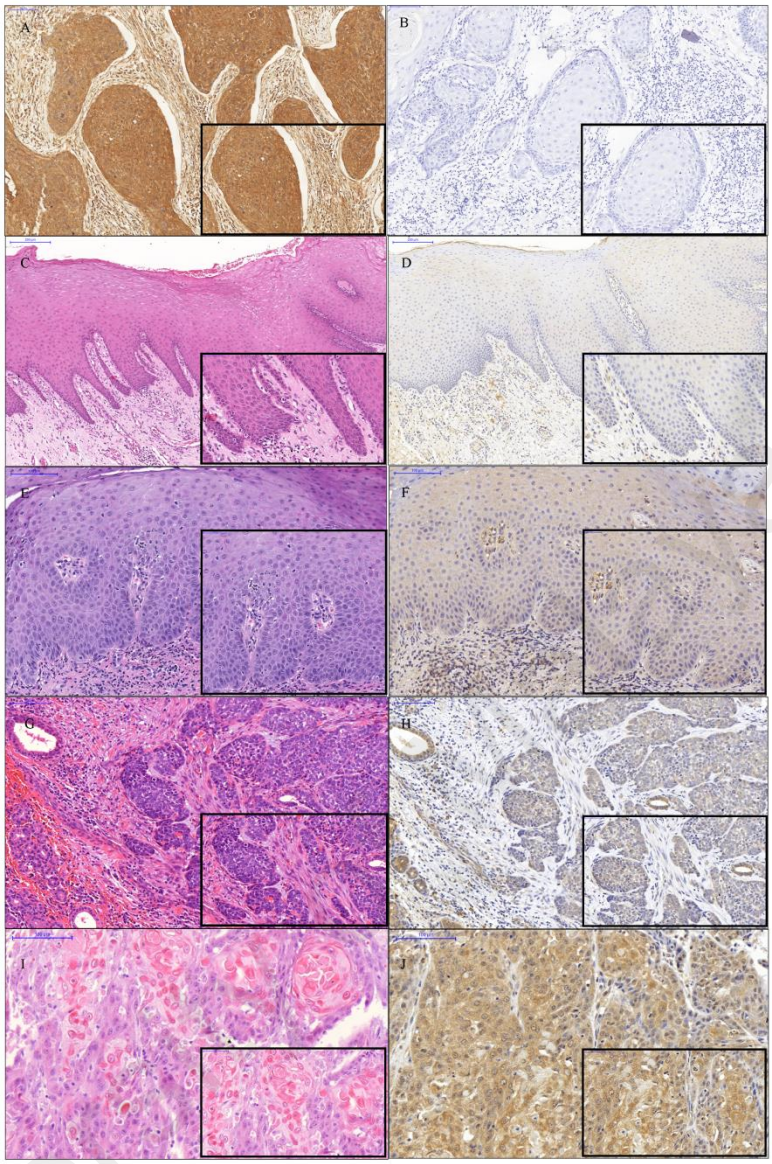


Figure 4.7: Immunohistochemistry of MMP13. The positive control: Ovarian cancer tissue (A) demonstrating expression of MMP13 protein. Strong cytoplasmic staining was observed within ovarian cancer tissue (Magnification 800x and 1600x). The negative control: OSCC tissue (B) demonstrating lack of staining when primary antibody is omitted. The negative control showed the negative cytoplasmic and nuclear staining within OSCC tissue (Magnification 800x and 1600x). Non-cancer oral mucosa (C) H&E stain (Magnification 400x and 1600x); (D) anti-MMP13 antibody immunostain was negative in the non-cancer oral mucosa (Magnification 400x and 1600x). OED tissue (E) H&E stain (Magnification 800x and 1600x); (F) anti-MMP13 antibody showed weak to moderate immunostaining in the cytoplasm of the OED cells (Magnification 800x and 1600x). OSCC (G) H&E stained (Magnification 800x and 1600x); (H) anti-MMP13 antibody immunostaining showed low expression in the cytoplasm of the epithelial tumour cells; OSCC (I) H&E stained (Magnification 800x and 1600x); (J) anti-MMP13 antibody immunostaining showed high expression in the cytoplasm of the epithelial tumour cells (Magnification 800x and 1600x).

4.3.1.2 Association of MMP13 mRNA expression with clinico-pathologic parameters and survival

Expression of MMP13 mRNA was significantly higher in OSCCs compared with non-cancer oral mucosa samples ($p < 0.005$), but it had no significant association with clinico-pathologic factors. The follow-up time for patients that were used for analysis of MMP13 mRNA expression ranged from 1 to 52 months (mean: 17.71 months, median: 13.0 months). Three-year survival rates for low and high mRNA expression of MMP13 were 57.76% and 17.45%, respectively. The MMP13 mRNA expression showed significant correlation with poor prognosis ($p = 0.016$) in Kaplan-Meier analysis (Figure 4.8). In multivariate Cox regression analysis, the expression of MMP13 mRNA remained as a significant prognostic factor for survival after adjustment for age, gender, risk habits and clinico-pathologic parameters (tumour sites, lymph node metastasis and tumour staging) which are the common confounding factors in OSCC (HRR = 2.23, 95% CI 1.015-4.896, $p = 0.046$) (Table 4.14).

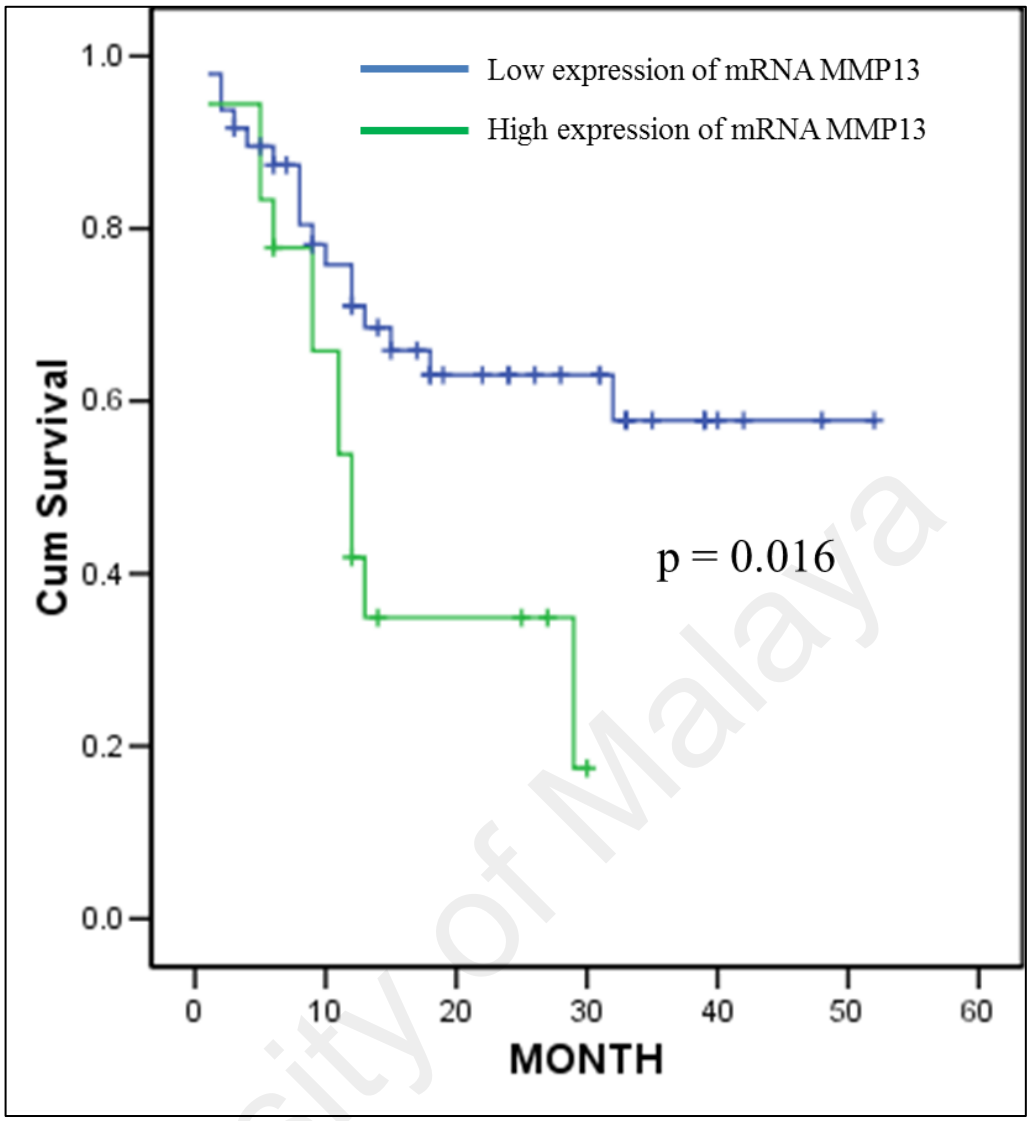


Figure 4.8: Overall survival curves were analyzed according to *MMP13* mRNA expression using Kaplan-Meier estimate with log-rank test.

Table 4.14: Multivariate Cox regression model analysis of *MMP13* mRNA expression in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		68			
mRNA expression of MMP13	Low	50 (73.5)	1.00†	1.015-4.896	**0.046**
	High	18 (26.5)	2.23		
Gender	Male	24 (35.3)	1.00†	0.388-2.806	0.933
	Female	44 (64.7)	1.043		
Age (years)	< 45	12 (17.6)	1.00†	0.397-3.009	0.864
	≥ 45	56 (82.4)	1.092		
Smoking	No	44 (64.7)	1.00†	0.240-2.071	0.524
	Yes	24 (35.3)	0.704		
Drinking	No	51 (75.0)	1.00†	0.391-2.341	0.922
	Yes	17 (25.0)	0.956		
Betel quid chewing	No	40 (58.8)	1.00†	0.652-3.718	0.319
	Yes	28 (41.2)	1.557		
Tumour site	Non-tongue*	38 (55.9)	1.00†	0.516-2.933	0.640
	Tongue	30 (44.1)	1.230		
Lymph node metastasis	Negative	33 (48.5)	1.00†	1.028-20.275	**0.046**
	Positive	35 (51.5)	4.565		
pTNM Staging	Early	22 (32.4)	1.00†	0.339-13.469	0.419
	Advanced	46 (67.6)	2.137		

CI: confidence interval
*****Buccal mucosa, gingiva, lip, floor of mouth, palate
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sites, lymph node metastasis and pathological tumour staging]

4.3.1.3 Definition of Cut-off Score for MMP13 protein expression in OSCC

Receiver operating characteristic (ROC) curve analysis was performed based on the results of IHC evaluation. Results showed that ROC curve analysis for tumour staging has the shortest distance from the curve to the point (0.0, 1.0) (Table 4.15). Hence, the cut-off value for tumour staging was selected. The cut-off score for low MMP13 protein expression was set to < 3.50 and the counterpart as a high MMP13 protein expression.

Table 4.15: Area under the receiver operating characteristic curve (AUC) of MMP13 for each clinico-pathological feature.

Clinico-pathological parameters	AUC (95% CI)	p value
Lymph node metastasis (Yes vs. No)	0.565 (0.453-0.677)	0.264
Tumour staging (Advanced vs. Early)	0.606 (0.486-0.725)	0.076
Tumour sizes (T1 and T2 vs. T3 and T4)	0.552 (0.439-0.664)	0.378
Survival (death vs. alive)	0.525 (0.409-0.642)	0.658

4.3.1.4 Association of MMP13 protein expression (IHC) with clinico-pathologic parameters and survival

A total of 103 OSCC samples were subjected to IHC. Results showed that high level of MMP13 protein expression was significantly associated with lymph node metastasis (LNM positive: 90.90% vs LNM negative: 70.2%, p = 0.011) and tumour staging (Advanced stage: 88.9% vs Early stage: 63.2%, p = 0.002) (Table 4.16). For MMP13 protein expression, the follow-up time for patients ranged from 1 to 92 months (mean: 29.13 months, median: 20.5 months). Three-year survival rate for the high and low expression of MMP13 protein was 34.73% and 72.38%, respectively. Results of the overall survival rate analysis demonstrated a significant association between positive MMP13 protein expression and poor prognosis (p = 0.005) (Figure 4.9). High MMP13 protein expression showed a considerable trend as an independent prognostic factor towards unfavourable overall survival after adjustment with other clinico-pathological parameters such as tumour subsites, lymph node metastasis, tumour staging, the pattern of invasion and tumour differentiation (HRR = 2.84, 95% CI 0.922-8.768, p = 0.069) (Table 4.17).

Table 4.16: Association of MMP13 protein expression (IHC) with clinico-pathological parameters.

Variables	Category	No. of patients (%)	MMP13 expression (n, %)		
			Low level of expression	High level of expression	p value
		103	21 (20.4)	82 (79.6)	
Total					
Gender	Male	35 (34.0)	10 (28.6)	25 (71.4)	0.139
	Female	68 (66.0)	11 (16.2)	57 (83.8)	
Age (years)	< 45	11 (10.7)	3 (27.3)	8 (72.7)	0.691
	≥ 45	92 (89.3)	18 (19.6)	74 (80.4)	
Smoking	No	81 (78.6)	15 (18.5)	66 (81.5)	0.380
	Yes	22 (21.4)	6 (27.3)	16 (72.7)	
Drinking	No	71 (68.9)	13 (18.3)	58 (81.7)	0.435
	Yes	32 (31.1)	8 (25.0)	24 (75.0)	
Betel quid chewing	No	49 (47.6)	10 (20.4)	39 (79.6)	0.996
	Yes	54 (52.4)	11 (20.4)	43 (79.6)	
Tumour site	Non-tongue*	68 (66.0)	14 (20.6)	54 (79.4)	0.944
	Tongue	35 (34.0)	7 (20.0)	28 (80.0)	
Tumour size**	T1-T2	59 (58.4)	16 (27.1)	43 (72.9)	0.063
	T3-T4	42 (41.6)	5 (11.9)	37 (88.1)	
Lymph node metastasis**	Negative	57 (56.4)	17 (29.8)	10 (70.2)	**0.011**
	Positive	44 (43.6)	4 (9.1)	40 (90.9)	
pTNM Staging**	Early stage	38 (37.6)	14 (36.8)	24 (63.2)	**0.002**
	Advanced stage	63 (62.4)	7 (11.1)	56 (88.9)	
Pattern of invasion**	Cohesive	13 (15.5)	5 (38.5)	8 (61.5)	0.140
	Non-cohesive	71 (84.5)	13 (18.3)	58 (81.7)	
Differentiation**	Well	45 (44.1)	11 (24.4)	34 (75.6)	0.392
	Poor and Moderate	57 (55.9)	10 (17.5)	47 (82.5)	

*****Buccal mucosa, gingiva, lip, floor of mouth, palate
** Data missing
Significant p - values are highlighted in bold.

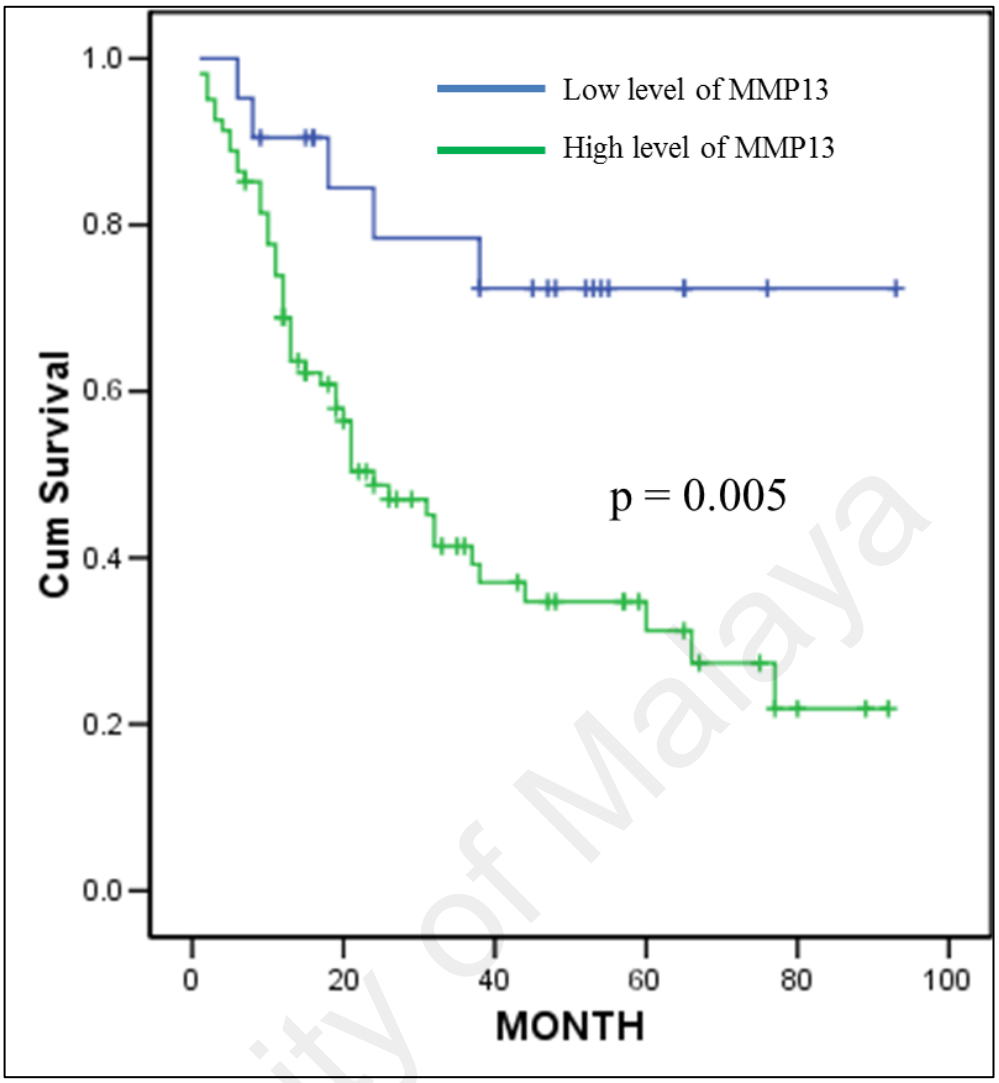


Figure 4.9: Overall survival curves were analyzed according to MMP13 protein expression (IHC) using Kaplan-Meier estimate with log-rank test.

Table 4.17: Multivariate Cox regression model analysis of MMP13 protein expression in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		103			
Protein expression of MMP13	Low	21 (20.4)	1.00†	0.922-8.768	0.069
	High	82 (79.6)	2.84		
Gender	Male	35 (34.0)	1.00†	0.366-2.530	0.938
	Female	68 (66.0)	0.96		
Age (years)	< 45	11 (10.7)	1.00†	0.127-2.997	0.548
	≥ 45	92 (89.3)	0.62		
Smoking	No	81 (78.6)	1.00†	0.150-2.017	0.367
	Yes	22 (21.4)	0.55		
Drinking	No	71 (68.9)	1.00†	0.376-1.656	0.531
	Yes	32 (31.1)	0.79		
Betel quid chewing	No	49 (47.6)	1.00†	0.195-1.178	0.109
	Yes	54 (52.4)	0.48		
Tumour site	Non-tongue*	68 (66.0)	1.00†	0.306-1.785	0.502
	Tongue	35 (34.0)	0.74		
Lymph node metastasis***	Negative	57 (56.4)	1.00†	0.771-4.188	0.175
	Positive	44 (43.6)	1.80		
pTNM Staging***	Early	38 (37.6)	1.00†	0.662-4.683	0.257
	Advanced	63 (62.4)	1.76		
Pattern of invasion***	Cohesive	13 (15.5)	1.00†	0.839-10.374	0.090
	Non-cohesive	71 (84.5)	2.95		
Differentiation***	Well	45 (44.1)	1.00†	0.258-1.009	0.050
	Moderate and poor	57 (55.9)	0.51		

CI: confidence interval
*****Buccal mucosa, gingiva, lip, floor of mouth, palate
† Reference category
Significant p - values are highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking) and clinico-parameters [tumour subsites, lymph node metastasis, tumour staging, pattern of invasion and pathological tumour differentiation]
*** Data missing

4.3.2 ISG15

4.3.2.1 ISG15 gene copy number, mRNA and protein expression in OSCC

Amplification of ISG15 was identified in 5.33% of the OSCC samples (4 out of 75) in chromosome 1p36.33 using array CGH (Table 4.2). In line with this, the ISG15 mRNA was found to be expressed significantly at a high level in 87.5% of the OSCC samples (56 out of 64 samples) with an average gene expression fold change of 46.03 compared to the non-cancer oral mucosa samples with fold change of 1 ($p = 0.001$) (Figure 4.10). In the current study, protein expression of ISG15 was examined by IHC analysis. Hepatocellular cancer tissue was used as the positive control for immunohistochemistry of ISG15 protein expression. The positive control tissue demonstrated a strong nuclear and cytoplasmic staining of the tumour cells (Figure 4.11). Moreover, the negative control (OSCC tissue) for immunohistochemistry showed an absence of cytoplasmic and nuclear staining (Figure 4.11). ISG15 was expressed in all OSCC and OED samples. IHC analysis clearly demonstrated that ISG15 protein expression was specifically detected in OED and OSCC indicating that this gene is likely to be associated with both OED and OSCC. In IHC analysis of ISG15 protein, the epithelial cells of non-cancer oral mucosal tissues showed a negative staining. A strong nuclear and cytoplasmic expression of anti-ISG15 antibody was observed in all layers of the OED epithelium. More than 40% of OSCCs displayed a strong nuclear and cytoplasmic staining of the epithelial tumour cells. The expression of ISG15 protein was statistically different between OSCC and non-cancer oral mucosal tissues ($p < 0.05$) in contrast to OSCCs and OEDs. A representative haematoxylin and eosin (H&E) and IHC stained image for protein expression group of ISG15 is shown in Figure 4.11.

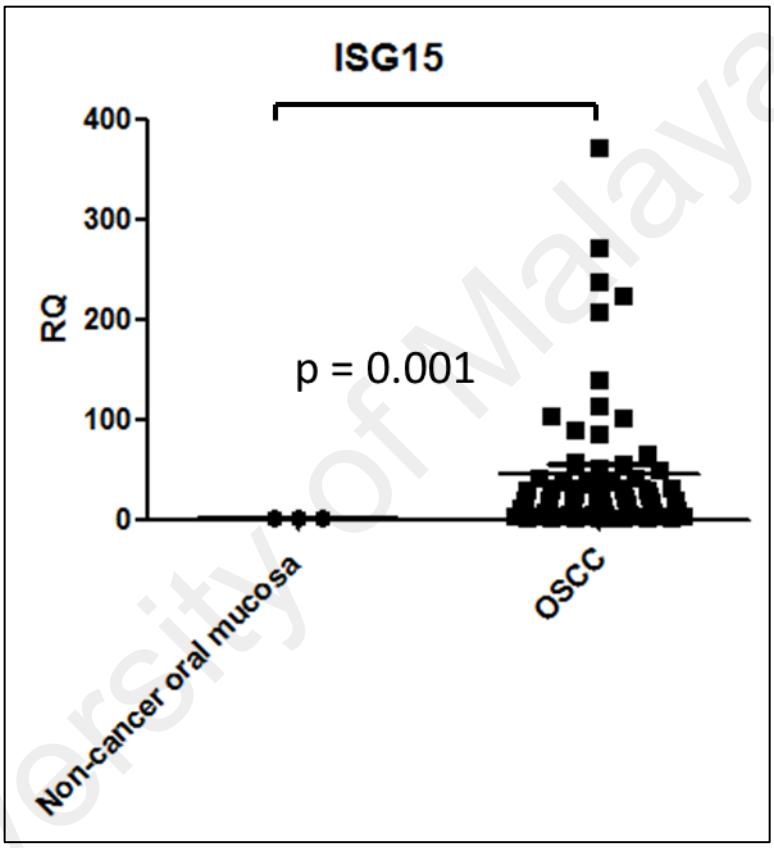


Figure 4.10: Average gene expression fold change of ISG15 in OSCC (n = 64) compared to normal oral mucosa sample (n = 5). Gene expression fold change of ISG15 was 46.03 while the gene expression between OSCC and non-cancer oral mucosa was statistically different ($p < 0.005$). The RQ for non-cancer oral mucosa of ISG15 was 1 and used for normalization.

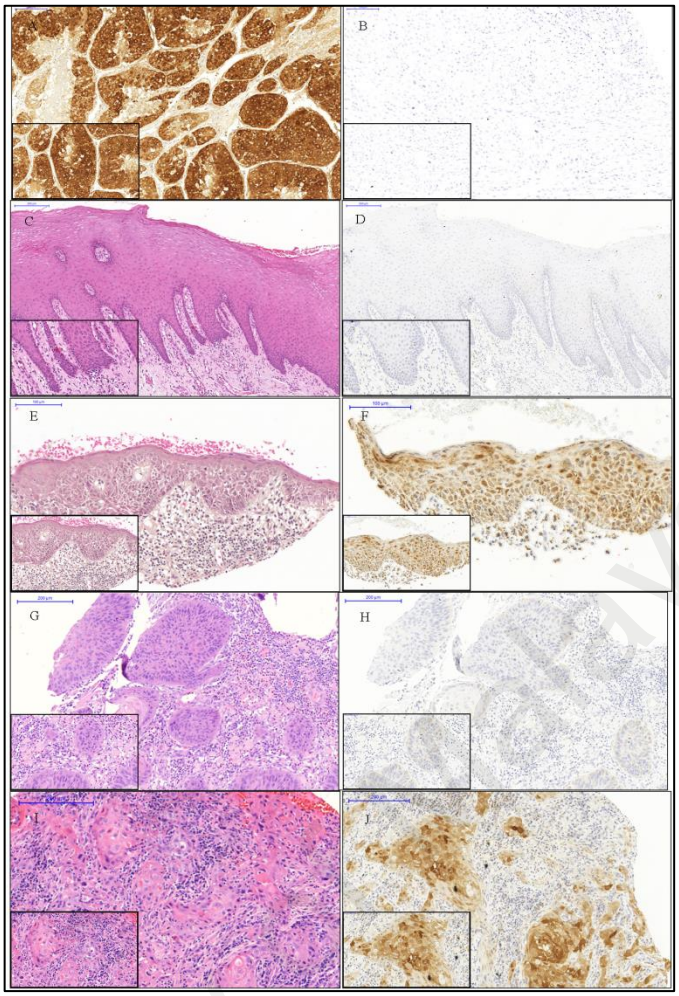


Figure 4.11: Immunohistochemistry of ISG15. The positive control: Hepatocellular cancer tissue (A) demonstrating expression of ISG15 protein. Strong cytoplasmic and nuclear staining was observed within hepatocellular cancer tissue (Magnification 800x and 1600x). The negative control: OSCC tissue (B) demonstrating lack of staining when primary antibody is omitted. The negative control showed the negative cytoplasmic and nuclear staining within OSCC tissue (Magnification 800x and 1600x). Non-cancer oral mucosa (C) H&E stain (Magnification 400x and 1600x); (D) anti-ISG15 antibody immunostain was negative in the non-cancer oral mucosa (Magnification 400x and 1600x). OED tissue (E) H&E stain (Magnification 800x and 1600x); (F) anti-ISG15 antibody showed strong nuclear and cytoplasmic expression of anti-ISG15 antibody all layers of the OED epithelium (Magnification 800x and 1600x). OSCC (G) H&E stained (Magnification 800x and 1600x); (H) anti-ISG15 antibody immunostaining showed weak cytoplasmic expression of anti-ISG15 antibody in the epithelial tumour cells (Magnification 800x and 1600x); (I) H&E stained (Magnification 800x and 1600x); (J) anti-ISG15 antibody immunostaining showed strong nuclear and cytoplasmic expression of anti-ISG15 antibody in the epithelial tumour cells (Magnification 800x and 1600x).

4.3.2.2 Association of ISG15 mRNA expression with clinico-pathologic parameters and survival

Expression of ISG15 mRNA was significantly higher in OSCCs (n = 64) compared with non-cancer oral mucosa samples (n = 5) ($p < 0.005$), but it had no significant association with clinico-pathologic factors and prognosis. The association between ISG15 expression with clinico-pathological parameters and survival are included in Appendix K and L, respectively.

4.3.2.3 Definition of cut-off score for ISG15 protein expression in OSCC

ROC curve analysis was performed based on the results of IHC evaluation. Results showed that ROC curve analysis for tumour staging has the shortest distance from the curve to the point (0.0, 1.0) (Table 4.18). Hence, the cut-off value for tumour staging was selected. The cut-off score for low ISG15 protein expression was set to < 4.0 and the counterpart as a high ISG15 protein expression.

Table 4.18: Area under the receiver operating characteristic curve (AUC) of ISG15 for each clinico-pathological feature.

Clinico-pathological parameters	AUC (95% CI)	p value
Lymph node metastasis (Yes vs. No)	0.627 (0.471-0.783)	0.118
Tumour staging (Advanced vs. Early)	0.637 (0.463-0.810)	0.106
Tumour sizes (T1 and T2 vs. T3 and T4)	0.499 (0.339-0.658)	0.986
Survival (death vs. alive)	0.615 (0.456-0.774)	0.153

4.3.2.4 Association of ISG15 protein expression with clinico-pathologic parameters and survival

Results showed that high level of ISG15 expression was significantly associated with gender (Female: 76.9% vs Male: 23.1%, p = 0.031), alcohol drinking habit (Yes: 19.2% vs No: 80.8%, p = 0.026), lymph node metastasis (LNM positive: 61.5% vs LNM negative: 38.5%, p = 0.004) and tumour staging (Advanced stage: 84.6% vs Early stage: 15.4%, p = 0.005) (Table 4.19). The follow-up time for the patients ranged from 3 to 93 months (mean: 36.7 months, median: 24.0 months). The 3- and 5- years survival rates for the high level of ISG15 expression were 38.5% and 25.2%, respectively whereas for the low level of ISG15 expression, it was 67.3% and 55.6%, respectively. Using the Kaplan-Meier survival curve analysis, it was demonstrated that the high level of ISG15 expression seems to be a significant predictor of poor overall survival (p = 0.029), Figure 4.12. To investigate whether ISG15 protein expression could predict the poor outcome of OSCC, multivariate Cox regression analysis were conducted to examine the effect of ISG15 in OSCC by additionally adjusting for age, gender, risk habits, and clinico-pathological parameters such as tumour site, tumour sizes, lymph node status, tumour staging, pattern of invasion and differentiation (Table 4.20). The analysis showed over-expression of ISG15 was not an independent prognostic factor. However, the hazard risk of protein expression of ISG15 in OSCC is 1.5 fold towards death (HRR = 1.475; 95% CI 0.484-4.491; p = 0.494).

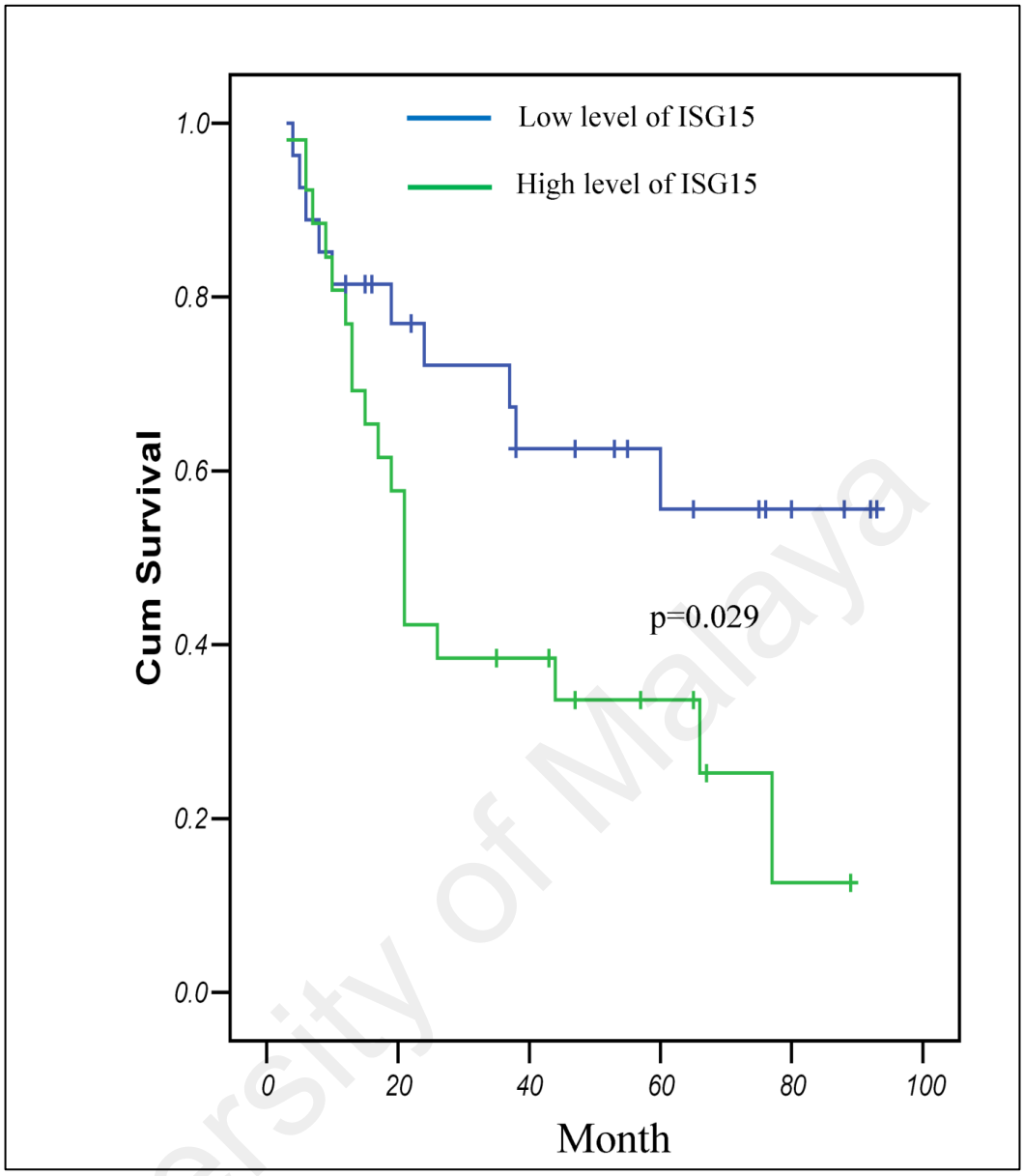


Figure 4.12: Overall survival curves were analyzed according to ISG15 protein expression using Kaplan-Meier estimate with log-rank test.

Table 4.19: Association of ISG15 protein expression with clinico-pathological parameters.

Variables	Category	No. of samples (%)	Low expression of ISG15	High expression of ISG15	
			No. of samples (%)	No. of samples (%)	p value
Total		53			
Gender	Male	20 (37.7)	14 (51.9)	6 (23.1)	**0.031**
	Female	33 (62.3)	13 (48.1)	20 (76.9)	
Age (years)	< 45	4 (7.5)	3 (11.1)	1 (3.8)	0.610
	≥ 45	49 (92.5)	24 (88.9)	25 (96.2)	
Smoking	No	42 (79.2)	20 (74.1)	22 (84.6)	0.344
	Yes	11 (20.8)	7 (25.9)	4 (15.4)	
Drinking	No	35 (66.0)	14 (51.9)	21 (80.8)	**0.026**
	Yes	18 (34.0)	13 (48.1)	5 (19.2)	
Betel quid chewing	No	23 (43.4)	15 (55.6)	8 (30.8)	0.069
	Yes	30 (56.6)	12 (44.4)	18 (69.2)	
Tumour site	Tongue	19 (35.8)	10 (37.0)	9 (34.6)	0.854
	Non-tongue*	34 (64.2)	17 (63.0)	17 (65.4)	
Tumour sizes	T1-T2	28 (52.8)	16 (59.3)	12 (46.2)	0.339
	T3-T4	25 (47.2)	11 (40.7)	14 (53.8)	
Lymph node status	Negative	31 (58.5)	21 (77.8)	10 (38.5)	**0.004**
	Positive	22 (41.5)	6 (22.2)	16 (61.5)	
pTNM Staging	Early stage	18 (34.0)	14 (51.9)	4 (15.4)	**0.005**
	Advanced stage	35 (66.0)	13 (48.1)	22 (84.6)	
Differentiation	Poor and Moderate	32 (60.4)	14 (51.9)	18 (69.2)	0.196
	Well	21 (39.6)	13 (48.1)	8 (30.8)	

*****Buccal mucosa, gingiva, lip, floor of mouth, palate
Significant p - value were highlighted in bold.

Table 4.20: Multivariate Cox regression model analysis of ISG15 protein expression in OSCC overall survival.

Variables	Category	No. of patients (%)	Multivariate Logistic regression**		
			HRR	95% CI	p value
Total		103			
Protein expression of ISG15	Low	27 (48.2)	1.00†		0.494
	High	26 (41.8)	1.475	0.484-4.491	
Gender	Male	20 (37.7)	1.00†		0.625
	Female	33 (62.3)	0.730	0.206-2.581	
Age (years)	< 45	4 (7.5)	1.00†		0.632
	≥ 45	49 (92.5)	0.638	0.101-4.013	
Smoking	No	42 (79.2)	1.00†		0.159
	Yes	11 (20.8)	0.301	0.057-1.598	
Drinking	No	35 (66.0)	1.00†		0.289
	Yes	18 (34.0)	0.589	0.222-1.567	
Betel quid chewing	No	23 (43.4)	1.00†		**0.035**
	Yes	30 (56.6)	0.276	0.083-0.915	
Tumour site	Non-tongue*	34 (64.2)	1.00†		0.547
	Tongue	19 (35.8)	0.715	0.240-2.128	
Tumour sizes	T1-T2	28 (52.8)	1.00†		0.276
	T3-T4	25 (47.2)	1.854	0.610-5.634	
Lymph node metastasis	Negative	31 (58.5)	1.00†		0.059
	Positive	22 (41.5)	3.104	0.956-10.076	
pTNM Staging	Early	18 (34.0)	1.00†		0.969
	Advanced	35 (66.0)	1.033	0.204-5.231	
Pattern of invasion	Cohesive	6 (11.3)	1.00†		0.142
	Non-cohesive	47 (88.7)	5.656	0.559-57.177	
Differentiation	Well	32 (60.4)	1.00†		0.063
	Moderate and poor	21 (39.6)	0.426	0.173-1.047	

CI: confidence interval
*****Buccal mucosa, gingiva, lip, floor of mouth, palate
† Reference category
Significant p - value were highlighted in bold.
**Multivariate logistic regression analysis was applied to adjust the confounders [age, gender, risk habits (cigarette smoking, betel quid chewing and alcohol drinking)] and clinico-pathologic parameters [tumour sites, lymph node metastasis and pathological tumour staging, pattern of invasion and differentiation]

Objective 4: To investigate the functional roles of a putative cancer gene using RNAi technique in OSCC cell lines.

4.4 Roles of a putative cancer gene using RNA interference (RNAi) technique in OSCC cell lines model

Network analysis using IPA software revealed a putative novel gene, namely ISG15 involved in network signalling that linked with cell death and survival, cellular movement, cellular development. This gene was prioritized for further evaluation and characterization in OSCC by quantification of mRNA and protein expression levels as well as *in vitro* functional analysis. ISG15 was selected on the basis of that its role in oral cancer was poorly understood and literature suggested a high probability of involvement in tumourigenesis.

4.4.1 Knockdown of ISG15 in oral cancer cell line ORL-48T, ORL-188T and ORL-204T

Initially, ISG15 expression at the protein level was evaluated in OSCC cells. Western blot showed ISG15 to be well-expressed in OSCC cells. To explore the role of ISG15 on oncogenic activities and treatment response in OSCC cells, siRNA was employed to inhibit endogenous ISG15 expression in OSCC cell lines. ISG15 protein expression was reduced by ISG15 siRNA in OSCC cells compared with cells treated with non-targeting siRNA control in all three OSCC cell lines namely ORL-48T, ORL-204T and ORL-188T (Figure 4.13). Taken together, these results showed successful knockdown of ISG15 in these three OSCC cell lines.

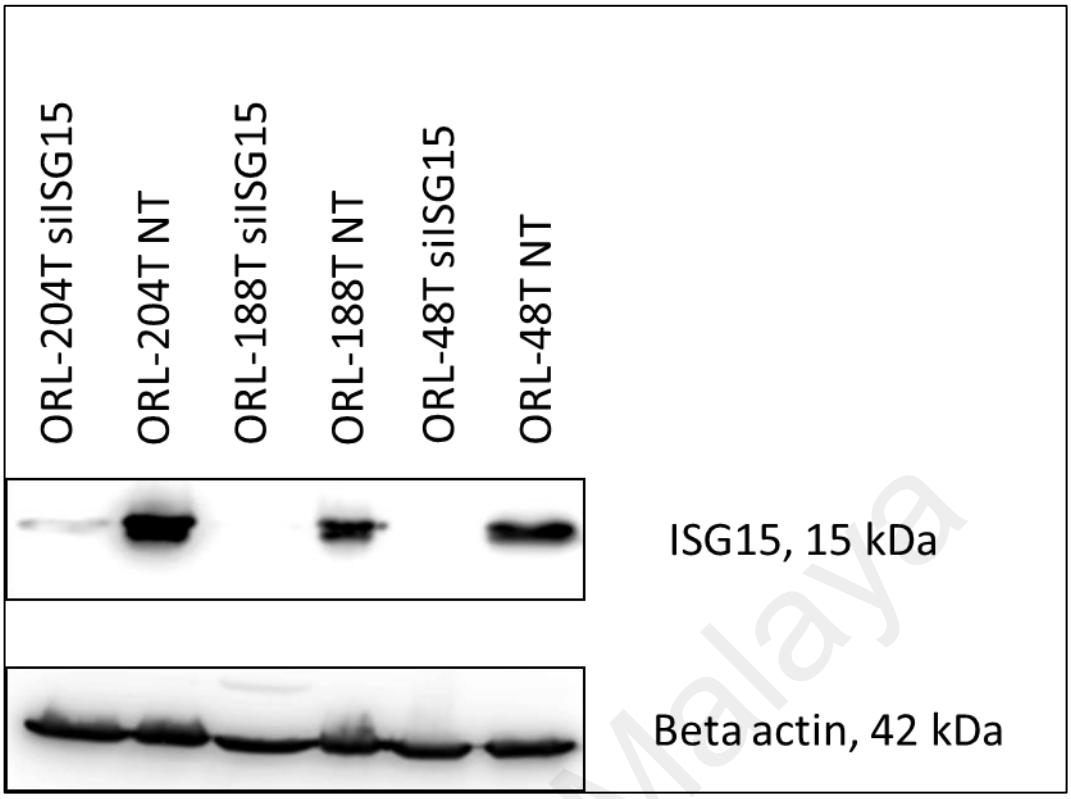


Figure 4.13: ISG15 protein was reduced by ISG15 siRNA in ORL-48T, ORL-188T and ORL-204T OSCC cells compared with non-targeting negative control siRNA.

4.4.2 *In vitro* functional assays to determine the effects of ISG15 expression in oral cancer cells

4.4.2.1 The role of ISG15 in cell proliferation

The role of ISG15 in promoting cell proliferation was determined using MTT cell proliferation assay on all three cell lines namely ORL-48T, ORL-204T and ORL-188T, cells transfected either with non-targeting siRNA control or ISG15 siRNA. The assay was conducted three times in triplicate to confirm the results. Proliferating cells, as determined by absorbance, were significantly decreased in all three cell lines with siISG15 transfection, as compared with negative control cells ($p = 0.0001$) at 24, 48, 72, 96, 120 and 144 hours (Figure 4.14). This observation was further supported by another proliferation assay using RTCA technology. The ISG15 gene knockdown in three OSCC cell lines demonstrated a decrease in the number of viable adherent cells (represented by cell index), compared to the non-targeting siRNA control cells in 30 hours. The doubling time for ORL-48T, transfected with non-targeting siRNA control and ISG15 siRNA were 10.79 ± 0.45 hours and 16.74 ± 1.29 hours, respectively. As for ORL-204T, the doubling time for the non-targeting siRNA control and ISG15 siRNA were 15.54 ± 0.68 hours and 23.11 ± 2.19 hours, respectively. Lastly, the doubling time for the non-targeting siRNA control and ISG15 siRNA transfected ORL-188T cells were 16.40 ± 0.64 hours and 26.20 ± 1.77 hours, respectively (Figure 4.14).

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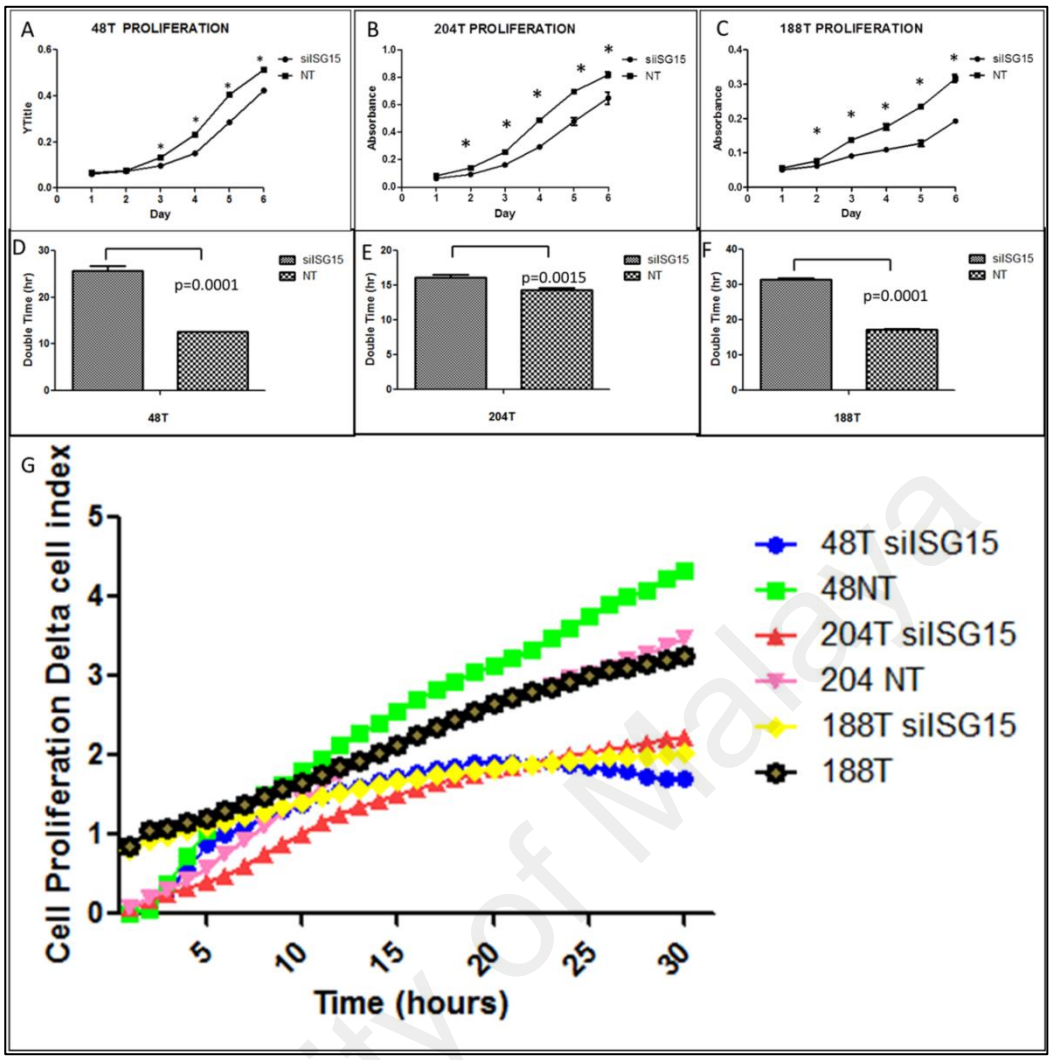


Figure 4.14: Effect of ISG15 on proliferation of OSCC cells. There was a significant difference in the cell proliferation among the non-targeting siRNA control and siRNA targeting ISG15 in (A) ORL-48T, (B) ORL-204T (C) ORL-188T cells. The doubling time was observed significantly different among the non-targeting siRNA control and siRNA targeting ISG15 in (D) ORL-48T, (E) ORL-204T (F) ORL-188T cells. The growth curve between the non-targeting siRNA control and siRNA targeting ISG15 obtained from RTCA was shown in Figure 4.15 (G). Data are expressed as mean ± SEM; *p < 0.05 compared to non-targeting siRNA control. Assays were carried out in triplicate.

4.4.2.2 Effect of the combination of chemotherapeutic agent with ISG15 silencing on Caspase 3/7 activation

The IC_{50} of cisplatin in ORL-48T, ORL-204T and ORL-188T was identified using MTT assay and there were 16 µM, 35 µM and 10 µM, respectively. The respective IC_{50} of the cisplatin were used for subsequent treatments. In order to gain insight into the mechanism by which ISG15 silencing enhanced drug cytotoxicity, the activity of the effector caspase 3 and 7, crucial components of the apoptotic cell death was determined.

In this study, MTT cell cytotoxicity assay and luminescent intensity for the caspase 3/7 activity was measured in OSCC cells after 48 hours of siRNA transfection of ISG15 followed by 48 hours of cisplatin treatment. The MTT assay showed that the combination of the ISG15 silencing and cisplatin treatment resulted in a reduction of cell viability compared with the non-targeting siRNA control OSCC cell lines treated with cisplatin alone (Figure 4.15) which was statistically significant, indicating that silencing of ISG15 with enhanced cisplatin induced cell death in OSCC cells. However, the reduction of the cell viability is more obvious particularly in ORL-188T compared to ORL-48T and ORL-204T. Comparing caspase 3/7 activity in cells treated with cisplatin, cells with reduced ISG15 by siRNA treatment also had increased activity significantly suggesting that cell death was measured with the MTT assay is likely to be apoptosis in OSCC cells (Figure 4.15).

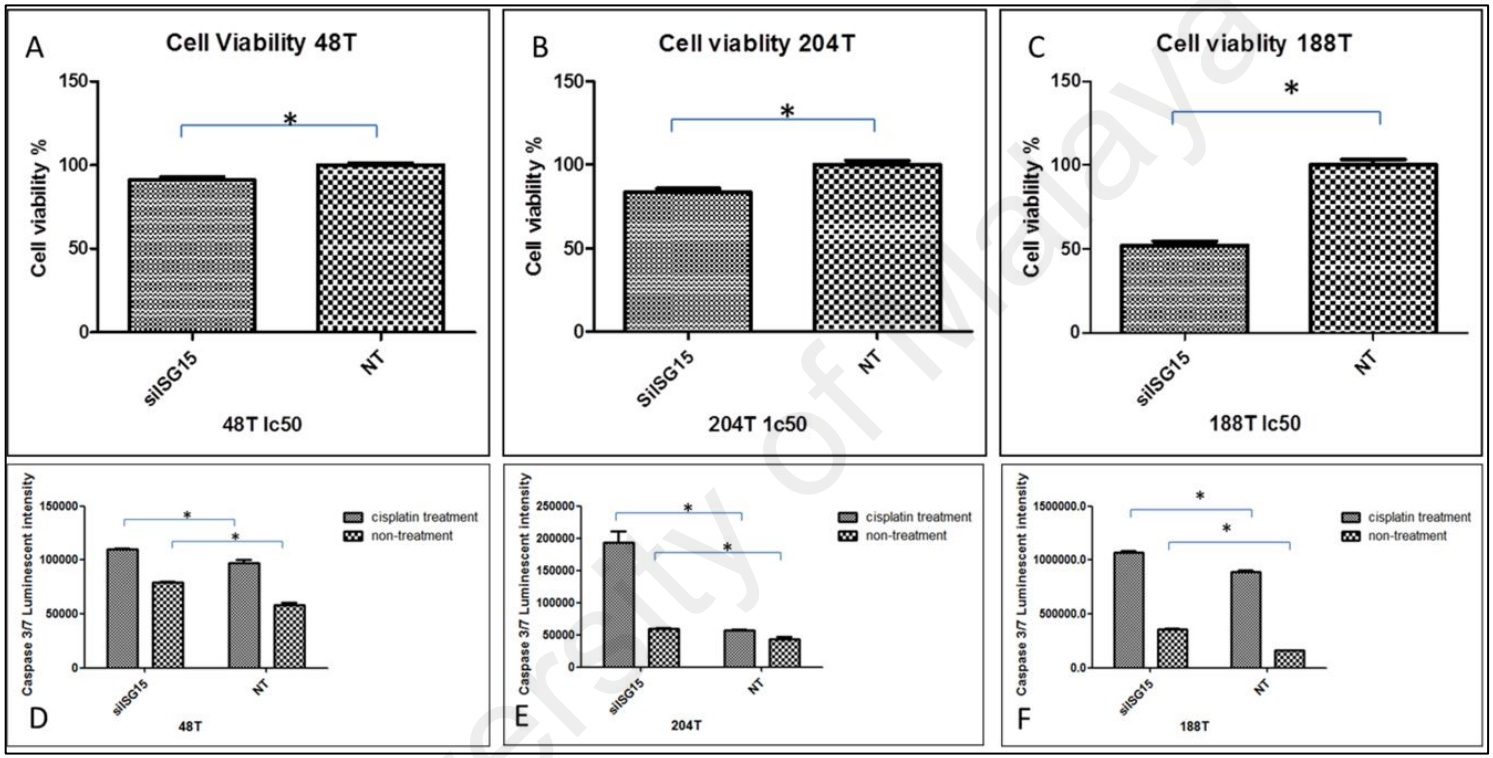


Figure 4.15: Effect of ISG15 silencing with cisplatin treatment on cell viability and caspase 3/7 activation of OSCC cells. Cell viability assay showed that the combination of the ISG15 silencing and cisplatin treatment resulted in a significant reduction of the absorbance compared with the non-targeting siRNA control OSCC cell lines that treated with cisplatin (A-C). The caspase 3/7 luminescent assays showed that the combination of the ISG15 silencing and cisplatin treatment resulted in higher levels of caspase 3/7 activity as compared to those obtained for cells only transfected with ISG15 siRNAs significantly (D-E). Data are expressed as mean ± SEM; *p < 0.05 compared to non-targeting siRNA control. Assays were carried out in duplicate for caspase 3/7 luminescent assays and triplicate for MTT assay

4.4.2.3 Effect of ISG15 on cell migration and invasion

The migratory effect of ISG15 on oral cancer cell was evaluated using wound healing assay and transwell migration method. Compared to the migratory ability of the control group in the cell migration assay (wound healing assay), the migratory activity of the ORL-48T, ORL-204T and ORL-188T cells were inhibited significantly after ISG15-siRNA transfection (Figure 4.16). In transwell migration assay, ORL-48T, ORL-204T and ORL-188T cells with siISG15 transfection exhibited a significant reduction in cell migration compared to the non-targeting control cells. Similarly, ORL-48T, ORL-204T and ORL-188T cells with siISG15 transfection exhibited a significant reduction in cell invasion compared to the non-targeting siRNA control cells (Figure 4.17). The transwell migration and invasion assays were conducted three times in triplicate and duplicate, respectively to confirm the results.

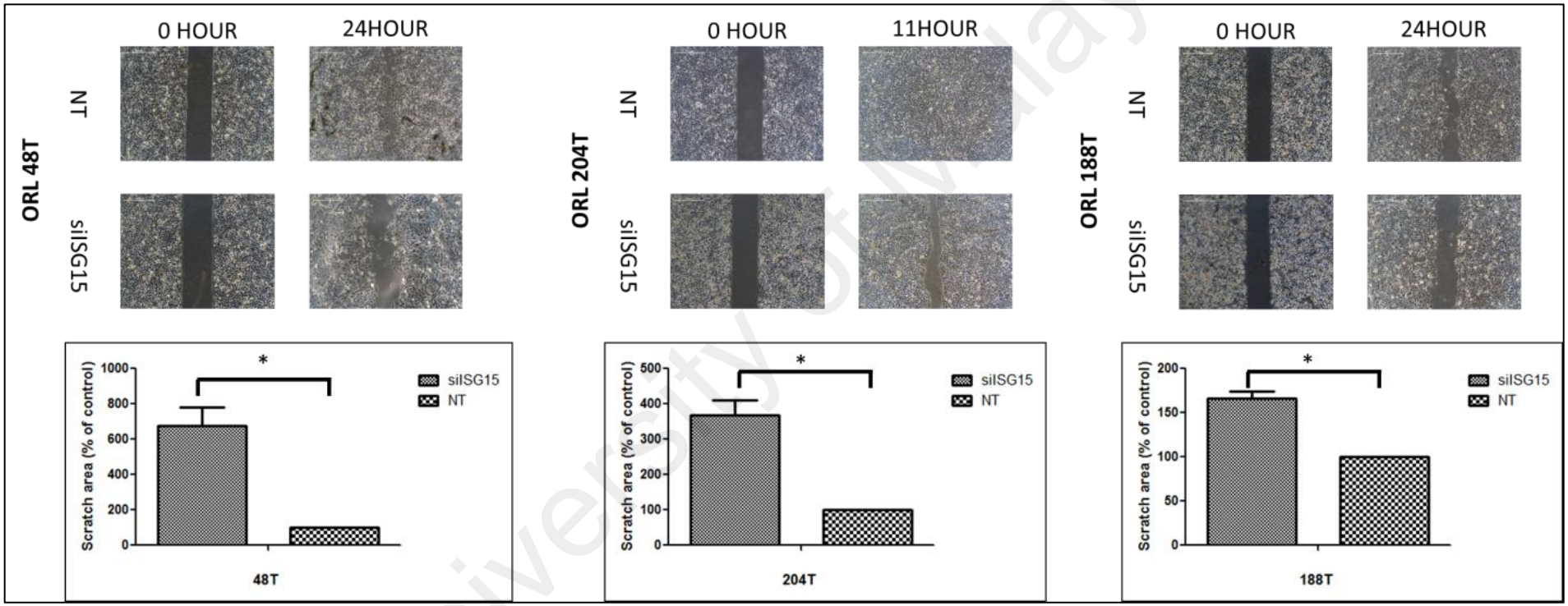


Figure 4.16: Effect of ISG15 on migration of OSCC cells using wound healing assay. Cellular migration was determined by wound healing scratch assay and analyzed by the Wimasis WimScratch software. The migratory ability of ORL-48T, ORL-204T and ORL-188T cells was reduced significantly with ISG15-siRNA transfection. Data are expressed as mean ± SEM; *p < 0.05 compared to non-targeting siRNA control. Assays were carried out in duplicate.

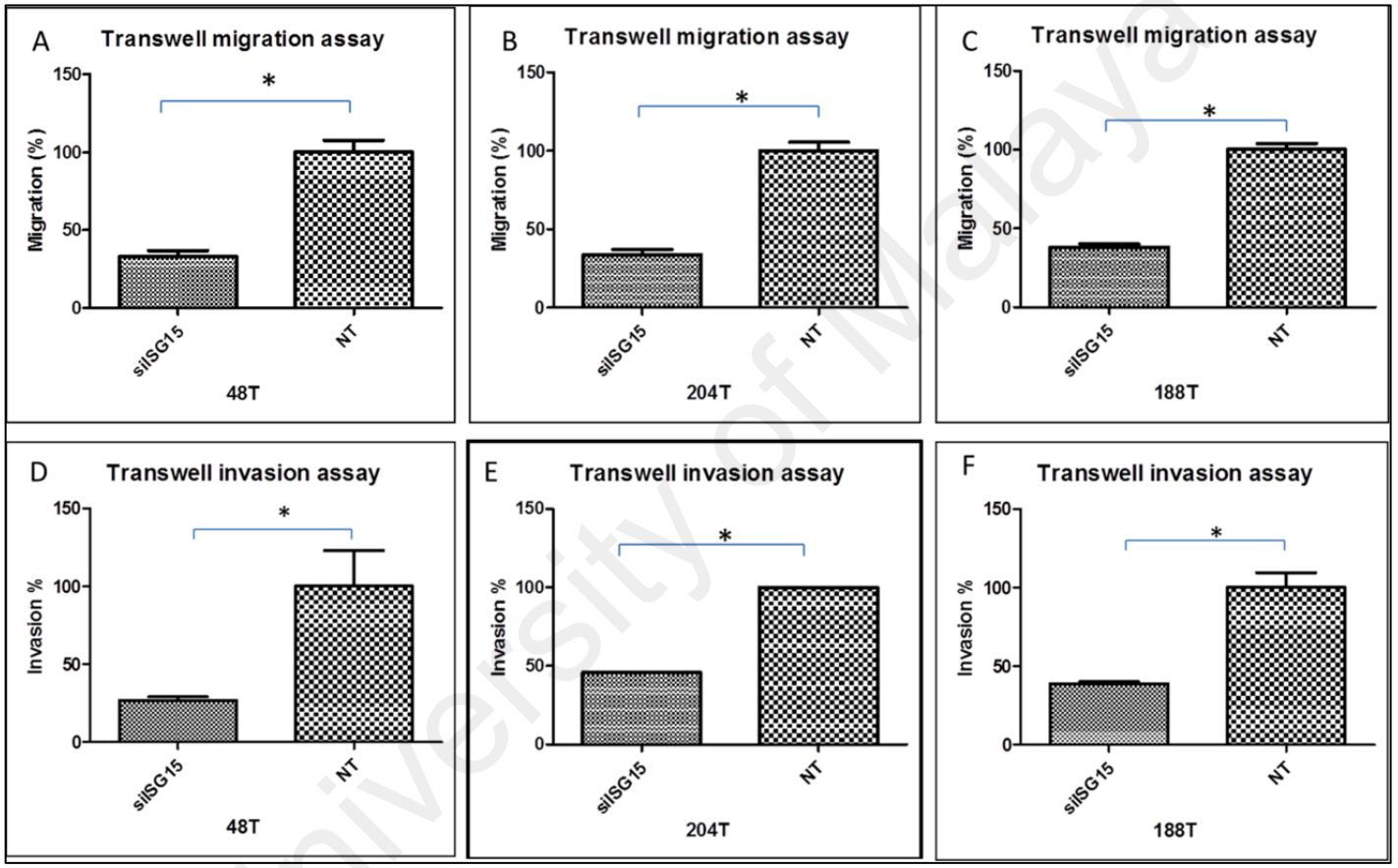


Figure 4.17: Effect of ISG15 on migration and invasion of OSCC cells using Transwell migration (A-C) and invasion assays (D-E). The results showed that there was a positive correlation between ISG15 expression and OSCC cell migration and invasion in ORL-48T, ORL-204T and ORL-188T cells lines. Data are expressed as mean ± SEM; *p < 0.05 compared to non-targeting siRNA control. Assays were carried out in triplicate for transwell migration assay and duplicate for transwell invasion assay.

CHAPTER 5: DISCUSSION

5.1 Determining and validating the CNAs of OSCC using array CGH and qPCR copy number assay

5.1.1 Copy number alterations of OSCC identified using array CGH

To the best of our knowledge, the TCGA consortium (TCGA, 2015) conducted the largest genome-wide profiling study on 172 OSCC samples including oral tongue, buccal mucosa, alveolar ridge, lip, floor of mouth and hard palate using array CGH technology followed by this study which detected wide CNA event among 75 OSCC samples. Due to high intra-tumoural and genetic heterogeneity of squamous cell carcinoma of the head and neck (HNSCC), only samples from oral cavities such as the tongue (excluding the base), buccal mucosa, alveolar ridge, retromolar trigone, hard palate, and floor of mouth were included. As such, the identified recurrent CNAs from this unique oral cavity SCC could provide new insights into the etiological basis of oral tumourigenesis.

CNAs can lead to the disruption of proto-oncogenes or tumour suppressor genes, as they have been determined to be the major determinant of poor prognosis in oral cancer (Ambatipudi *et al.*, 2011; Uchida *et al.*, 2011; Chen *et al.*, 2015). In this study, 28 amplifications and 3 deletions with a frequency of ≥ 5% (n = 75) were identified. Gains were more frequent than losses and were detected in chromosomes 8q, 7p, 5p, 11q, 3q, 10p and 1p while losses were observed in chromosomes 3p and 8p. This study confirms and adds to the earlier evidence of frequent CNAs that have been reported in the TCGA of oral cancer meta-analysis array CGH OSCC study and the International Cancer Genome Consortium (ICGC) which forms the most comprehensive integrative genomic

analysis of OSCC (ICGC, 2013; TCGA, 2015). In view of this, CNAs and the associated genes provide new insights into oral cancer biology and prognosis suggesting that shared and unique alterations of the CNAs accelerated progress involving prevention and therapy in OSCC.

The identification of high-frequency CNAs in chromosomes 3, 8, and 11 in this study reflects the existence of oncogenes and tumour suppressor genes that could be associated with poor prognosis in oral cancer (Bockmuhl *et al.*, 2000; Wreesmann *et al.*, 2004; Chen *et al.*, 2015). As such, further investigations on these chromosomes could provide enhanced prognosis predictions and anticancer strategies for OSCC patients. In this study, an amplification of 8q22.3-q23.1 was found to be the most frequent event, being present in 18.70% (n = 75) of all OSCCs. Genomic alterations in chromosome 8q have been frequently reported in OSCC, especially at an amplification of 8q22.3-q23.1, which is associated with disease-specific survival in human cancers such as OSCC and bladder cancer (Peng *et al.*, 2011). In addition, an amplification of chromosome 8q was found to be associated significantly with advanced stages in OSCC patients. This amplicon contains various proto-oncogenes including LRP12 and CTHRC1. The LRP12 gene belongs to the LDLR superfamily and according to Garnis *et al.* (2004b) suggest that this gene is more likely to behave like proto-oncogenes in oral tumourigenesis. Collagen triple helix repeat containing 1 (CTHRC1), a pro-migratory protein associated with tumour metastasis, was found to be over-expressed in several solid tumours, such as breast cancer and melanoma (Tang *et al.*, 2006). In this study, CTHRC1 was identified in the amplification of chromosome 8q22 and this observation is in agreement with the studies by Liu *et al.* (2013) and Tameda *et al.* (2014) who demonstrated that N-glycosylated CTHRC1 enhanced tumour cell migration and metastasis in oral tumourigenesis in an *in vitro* study. As such, the oncogenic

characteristic of this gene provides some basis for using this gene in cancer gene therapy in oral cancer management.

Losses in 3p26.3-3p26.1 and 3p21.31 were found to be the most frequent observations in the CNAs and the deletion of this region has been identified as a prognostic factor in OSCC patients. This region harbors the potential tumour suppressive related gene namely cell adhesion molecule with homology to L1CAM1 (CHL1), contactin 4 (CNTN4), and contactin 6 (CNTN6) as also evident in the recent OSCC genome-wide profiling study conducted by Chen *et al.* (2015). CHL1 is a member of the family of L1 neural cell adhesion molecules. It has been suggested that the loss of this gene in the early stages of cancer contributes to the growth and metastasis of human cancers (Senchenko *et al.*, 2011). Functional studies show that CHL1 is able to suppress the proliferation and invasion of tumour cells both *in vitro* and *in vivo* in breast tumourigenesis (He *et al.*, 2013). In combination with our results, these observations suggest that CHL1 is a candidate for the tumour suppressor gene associated with aggressiveness of OSCC. CNTN4 and CNTN6 were reported to be the cancer candidate gene for breast and colorectal cancer, respectively *via* somatic mutations screening using PCR in breast and colorectal cancer samples (Sjoblom *et al.*, 2006). Sjöblom *et al.* (2006) also reported that these genes were found to be involved in cellular adhesion and motility activities in tumourigenesis. Similarly, Chen *et al.* (2015) recently reported that CHL1, CNTN4, and CNTN6 were deleted in OSCC genome, and this has provided new options for diagnostic and therapeutic interventions in oral cancer.

In this study, the highest number of CNAs was observed on both arms of chromosomes 3 and 8, and both amplifications and deletions were detected on them. In fact, there were several oncogenes identified in chromosomes 3q and 8q and tumour suppressor genes in the 3p and 8p arm in oral cancers (Vincent-Chong *et al.*, 2016). These genes may play a role in oral tumourigenesis and can potentially be used in diagnosis and prognosis. Remarkably, the loss in 8p23.32 was one of the most frequent CNAs on the short arm of chromosome 8 (Vincent-Chong *et al.*, 2016). In this study, the deletion of chromosome 8p was found to be associated with advanced staging in OSCC. The focal region of 8p23.32, which harbors the CUB and sushi multiple domain protein 1 (CSMD1) genes, could be a region of interest for further investigation. CSMD1 is a candidate for the tumour suppressor gene that encodes a transmembrane protein that is involved in signal transduction in tumourigenesis (Kirkitadze & Barlow, 2001). Studies show that deletions and loss expressions of this gene were associated with poor survival and clinico-pathological parameters such as lymph node metastasis and tumour staging in several cancers (Tang *et al.*, 2012). The plausible reasons attributed to this tumour suppressive characteristic are its ability to inhibit tumour cell proliferation and migration and to induce apoptosis in tumourigenesis as demonstrated in *in vitro* and *in vivo* melanoma models. This provides the insight into the mechanism of oncogenesis and therapeutically actionable CNAs especially in this region for oral cancer.

Somatic CNAs in terms of amplification of chromosome 3q and deletions of chromosome 3p are well documented (Rowley *et al.*, 1996; Roz *et al.*, 1996). It appears that 3p26.3 is the target of deletion and that 3q27.1 is the target of amplification. Among these chromosomal regions, 3p26.3 on the short arm and 3q27.1 on the long arm appear to be regions of interest. In contrast to chromosome 8, it appears that copy number alterations on this chromosome are associated with tumour initiation and progression towards invasive cancers (Heselmeyer *et al.*, 1996; Salahshourifar *et al.*, 2014). A systematic review study that lead by Professor Rosnah and her team in Oral Cancer Research and Coordinating Center, Faculty of Dentistry, University of Malaya reported that segmental 3p deletions have been identified in oral potentially malignant diseases (OPMDs) and early stages of OSCC, whereas multiple segmental or broad 3p deletions have been detected in advanced stage tumours (Salahshourifar *et al.*, 2014). In addition, amplification of 3q was reported to be associated with advanced stages of OSCC, indicating that alterations in this region could be an important transition event in tumour progression to invasive OSCC (Oga *et al.*, 2001). The amplification of 3q13.32-q21.2 (MUC13), 3q25.31-q26.1 (CCNL1), 3q27.3-q28 (TP63), and 3q26.1-q26.31 (SKIL) were observed in this study and have been implicated in cancers (Yang *et al.*, 1999; Reed *et al.*, 2005; Sticht *et al.*, 2005; Chauhan *et al.*, 2009). Mucin 13 (MUC13) encodes a transmembrane mucin and studies have demonstrated over-expression of MUC13 gene enhanced tumour cell proliferation, migration, and invasion in *in vivo* and *in vitro* pancreatic and ovarian cancer models (Chauhan *et al.*, 2009; Chauhan *et al.*, 2012). Cyclin L1 (CCNL1) encodes a key regulator of pre-mRNA processing and is involved in G0 to G1 transition during the cell cycle (Iyer *et al.*, 1999; Berke *et al.*, 2001). Previous studies have shown that CCNL1 was highly amplified in a tongue SCC cell line and over-expressed in HNSCC primary samples (Redon *et al.*, 2002). In 2005, Sticht *et al.* (2005) noted that the amplification of CCNL1 acted as a clinical predictor

for advanced OSCC patients with lymph node metastasis and the fact that it remained as an independent prognosticator in OSCC suggests its potential role as a putative oncogene in head and neck cancers. P63 encodes a transcription factor, which plays an essential role in the development of epithelial tumourigenesis (Yang *et al.*, 1999). Studies have shown that the amplification of this gene has been identified in cervical cancer cell lines using array CGH technology. Apart from that, Lo Muzio *et al.* (2005) demonstrated that over-expression of the p63 protein was associated significantly with poor differentiation of OSCC and poor survival rates. SKIL is known as a negative regulator of TGF-beta signaling in tumourigenesis (Deheuninck & Luo, 2009). This amplified gene was reported in several cancers including head and neck cancers (Deheuninck & Luo, 2009). Studies show that reduced expression of this gene arrests tumour growth both *in vitro* and *in vivo* in lung and breast cancers (Zhu *et al.*, 2007). Similarly, SKIL can also function as a transcriptional activator, target the β-catenin pathway and enhance tumour cell survival, migration, and invasion in melanoma tumourigenesis (Reed *et al.*, 2005).

In this study, the co-amplification of chromosome 11q13.3 and 11q22.2 was found to be associated with advanced tumour staging in OSCC. The TCGA consortium showed co-amplification of the regions 11q13 and 11q22 in OSCC (TCGA, 2015). It is well known that the long arm of chromosome 11 harbors cancer-related genes that play an important role in pathogenesis and tumour development (Roh *et al.*, 2000; Beroukhim *et al.*, 2010). Several candidate oncogenes, such as CCND1, CTTN, ORAOV1, ANO1, and FADD, have been reported to promote tumour cell proliferation and evade tumour cell apoptosis, invasion, and migration in tumourigenesis (Hinds *et al.*, 1994; Patel *et al.*, 1998; Chen *et al.*, 2005; Jiang *et al.*, 2008; Ruiz *et al.*, 2012). The amplification of 11q13 was correlated with poor prognosis (Akervall *et al.*, 1995) and lymph node

metastasis in HNSCC (Rodrigo *et al.*, 2000), which implies that these driver gene candidates might be valuable as biomarkers in the prognosis and treatment planning for oral cancers. The results also show that the amplification of chromosome 11q22.2 and the genes that were mapped on this amplicon include BIRC2 and YAP1 and many MMPs (MMP-1, -3, -7, -8, -10, -12, -13, -20, and -27) suggesting the essential function of metalloproteinases in OSCC progression. As such, it seems that the aberration in chromosomes 11q is associated with the invasive behavior of tumours and poor prognosis.

The amplification of chromosome 7p12-22 has been identified in about 30% of OSCC and more than 40% of HNSCC samples from TCGA using SNP array CGH technology (Sheu *et al.*, 2009; TCGA, 2015). In this study, the amplification of chromosome 7p was associated with tumour sizes (T3-T4) and advanced tumour staging in OSCC. The candidate oncogenes harbored in this amplicon include EGFR, TWIST1, and HOXA genes. Among these oncogenes, EGFR has a high level of amplification in the OSCC TCGA sample (TCGA, 2015). EGFR is a transmembrane glycoprotein that includes an intracellular tyrosine kinase domain (Herbst, 2004). The activation of EGFR promotes tumour cell proliferation and evades apoptotic signals in tumourigenesis (Herbst, 2004). The amplification of TWIST1 and several of HOXA genes were identified in chromosome 7p22.3 in this study. TWIST1 is a transcription factor in which the high expression of this gene was associated with advanced stage and lymph node metastasis in OSCC with poor clinical outcomes. The inhibition of this gene suppressed cell metastasis ability in OSCC *in vitro* and *in vivo* using an orthotropic mouse model of metastatic OSCC (da Silva *et al.*, 2014). The implication of TWIST1 in cell migration is attributed primarily to its ability to contribute towards epithelial to mesenchymal transition (EMT) in oral tumourigenesis (da Silva *et al.*, 2014). HOX

genes encode homeodomain-containing transcription factors involved in the regulation of cellular proliferation and differentiation during embryogenesis. Bitu *et al.* (2012) reported that the inhibition of HOXA1 in oral squamous cell carcinoma (OSCC) induced a potent inhibition of cell proliferation in an *in vitro* OSCC model, and over-expression of HOXA1 remained as an independent prognosticator in OSCC. This highlights the need for further elucidation of the role of other members of the HOX family in OSCC.

In this study, the amplification of chromosome 9p21.1-13.3 was identified in 14.7% of OSCC samples recruited for genome-wide profiling using array CGH. The amplification of chromosome 9p especially 9p13 has been frequently reported in OSCC and is vital to the early stages of oral tumourigenesis (Snijders *et al.*, 2005; Ambatipudi *et al.*, 2011; Towle *et al.*, 2014). In this study, the amplification of chromosome 9p was associated significantly with lymph node metastasis, and this might be driven by the proto-oncogenes including CA9, VCP, DCTN3, and STOML2 harboured in this amplicon. Carbonic anhydrase 9 (CA9) is a glycoprotein and its activation plays an important role in a hypoxic microenvironment which allows tumour cell proliferation (Ivanov *et al.*, 1998). Previous studies demonstrate that CA9 expression was associated with poor survival in OSCC suggesting that this gene plays an important role in OSCC progression (Brockton *et al.*, 2012). VCP (p97) is a member of the ATPases associated with various cellular activities (AAA) super-families and is known to activate NF-κB signaling which enhances tumour cell proliferation and promotes tumour survival by evading apoptosis signals in tumourigenesis (Asai *et al.*, 2002; Yamamoto *et al.*, 2004). Stomatin-like protein 2 (STOML2), a member of the stomatin family, has been reported to be over-expressed in several types of human cancers (Song *et al.*, 2012). Song *et al.* (2012) demonstrated that this gene might play an important role in glioma invasion and

in modulation of the NF-κB signalling pathway. This has prompted studies on the potential of this gene as a therapeutic agent in the treatment of other cancers. Towle *et al.* (2014) demonstrated that the inhibition of these putative genes in OSCC cell lines suppressed tumour cell proliferation and suggested that the amplification of 9p13 is more likely to contribute to the aggressiveness of multiple oncogene candidates in oral tumourigenesis.

5.1.2 TaqMan copy number assay of LRP12, TPM2, EGFR, FSCN1, CCND1, CLPTM1L, CHL1 and CSMD1

CNAs identified from array CGH were further validated using the qPCR method. In this study, qPCR copy number analysis on LRP12, TPM2, EGFR, FSCN1, CCND1, CLPTM1L, CHL1 and CSMD1 genes was carried out. It identified a "genetic signature" consisting of the LRP12, CCND1, EGFR and TPM2 genes that could predict clinical outcomes and may ultimately facilitate patient-tailored selection of therapeutic strategies in oral cancer management.

The identification of this 'genetic signature' as a prognosticator for patients may have the potential to facilitate treatment decisions and allows for comprehensive approaches to be taken for patients with poor prognoses. This approach basically relies on the presence or absence of this genetic signature in patients in order to establish a personalized medicine strategy to be used in clinical application. This finding demonstrates that these genetic markers may be a useful tool in the clinical management of OSCC patients.

The genetic signatures identified in this study provide prognostic information beyond routine clinical utility by shedding light on the molecular mechanisms underlying aggressiveness in OSCC that could be targeted for cancer treatment. EGFR is a member of the epidermal growth factor family and has been shown to be associated with poor patient survival outcome as its downstream signalling contributes to tumor growth and metastasis (Zimmermann *et al.*, 2006; Tsui *et al.*, 2009). Aberrant expression of LRP12, an LDL receptor related protein, has also been found to over-express in OSCC, suggesting its role as a proto-oncogene in oral tumourigenesis (Garnis *et al.*, 2004b). In addition, TPM2, a member of the Tropomyosins superfamily, plays a major role in the

control of stress fibers of human epithelial cells and the differences in its expression has been demonstrated in malignant breast epithelial cell lines compared to 'normal' breast cell lines (Dube *et al.*, 2016). It is well documented that over-expression of CCND1 was an important oncongenic event in various cancer, including oral cancer. Over-expression of this gene was found to be associated with poor prognosis, lymph node metastasis and tumor staging (Lam *et al.*, 2000; Zhao *et al.*, 2014). CCND1 overexpression also promotes tumor cell proliferation through the downstream activation of the cyclin-dependent kinases (CDKs) CDK4 and CDK6 mechanisms, which serves to be a potential therapeutic target (Musgrove *et al.*, 2011).

5.2 Determining pathways and networks implicated in OSCC pathogenesis

Previously, pathway and network analyzes have been applied in various studies to filter the driver genes involve in the signalling pathways and to identify key regulators of cancer-related gene networks from the robust cancer data sets (Xu *et al.*, 2010; Li *et al.*, 2014b; Mutation Consequences and Pathway Analysis working group of the International Cancer Genome, 2015). In this study, several oncogenic signalling pathways were identified using the IPA pathway analysis, and the most significant signalling pathway was associated with Integrin-linked kinase (ILK) signalling. Integrin-linked kinase, a candidate oncogene, acts as a multifunctional serine/threonine kinase and its activation is involved in the regulation of cell proliferation, apoptosis, differentiation, migration, and epithelial-mesenchymal transition (EMT) (Que *et al.*, 2016). In this signalling pathway, the activated SNAI (8q11.2) identified in this genome-wide profiling study plays a role in the activation of the RhoGTPases and enhanced tumour cell motility *via* the cytoskeletal remodeling mechanism (Nieto, 2002). In this signalling pathway, the downstream effect of the phosphorylation activation of Akt suppresses the apoptosis signal and promotes tumour cell survival by

inhibiting Caspase-3/9 activities while inducing the over-expression of CCND1 (11q13.3) which promotes cell proliferation in tumourigenesis (Cordes & van Beuningen, 2003). This downstream target of VEGFB (11q13.3) in this signalling process also binds to PI3K and is therefore implicated in the regulation of anchorage-dependent cell growth and survival, cell-cycle progression EMT, invasion and migration, and cell metastasis in tumourigenesis (Legate *et al.*, 2006). Recently, Que *et al.* (2016) showed that targeting the ILK signalling pathway would suppress tumour cell proliferation, the adhesion and invasion ability in oral tumourigenesis and inhibit tumour growth, invasion, and metastasis in the *in vivo* model. These findings highlight that the ILK signalling pathway plays a novel role in oral tumourigenesis by regulating EMT associated genes and other downstream targets in this pathway. Thus, this signalling could be a promising targeted pathway for therapeutic intervention in oral cancer.

In this study, IPA also shows that the biological functions related to the CNAs associated genes included cell death and survival, cellular functions and maintenance, cellular development, cellular growth and proliferation, and cellular movement. These functions are basically involved in sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, and activating invasion and metastasis. All these hallmark cancer-associated functions are well-documented by Douglas Hanahan and Robert Weinberg in their review paper (Hanahan & Weinberg, 2000). The identification of the CCND1, an amplified gene harboured in 11q13.3 was involved in all of the findings and implies that its over-expression would lead to the characteristics of cancers by promoting the proliferation, migration, and invasion of tumour cells and the evasion of apoptosis signals in oral tumourigenesis. Cyclin D1 enhances tumour cell proliferation, migration, and invasion, and evades

apoptosis by the dys-regulation of CDK4/CDK6 activity, RB phosphorylation, and activation of E2F *via* the cyclin D–RB–E2F pathway in tumourigenesis (Musgrove *et al.*, 2011).

In this study, the IPA analysis of the CNAs associated genes revealed a number of significant biological annotations that related to tumour cell proliferation and evade apoptosis signals to promote cell survival and metastasis in tumourigenesis. Of these, the amplification of CCND1, RELA, TP63, and EGFR genes, which are in turn located on 11q13.3, 11q13.1, 3q28, and 7p11.2 was identified as having a central role in tumour cell proliferation, immortalization, and metastasis in oral tumourigenesis. The downstream effects of the recurrent CNAs in cancer cells could include a disruption of the oncogenes and tumour suppressor genes in the dys-regulated cell signalling pathways/networks. The interaction network between these four candidate oncogenes namely CCND1, RELA, TP63, and EGFR have been associated with several oncogenic pathways which are the PI3 Kinase/Akt signalling, NF-κB signalling, cell cycle control signalling, and MAPK/Erk ingrowth and differentiation signalling. The active growth factor (EGF) activated the EGFR through the binding of its extracellular domain leading to the downstream cascade activation of STAT 1 and 3, phosphatidylinositol 3-kinase PI3 Kinase/Akt and the RAS-MAPk/Erk signalling pathway which in turn lead to the translocation of NF-κB into the nucleus and induced CCND1 transcription (Bazley & Gullick, 2005; Song *et al.*, 2005; Williams, 2010). Apart from that, the binding of the RELA gene to NF-κB eventually induced the CCND1 expression which promotes tumour cell immortalization, proliferation, and metastasis in tumourigenesis. The activation of ERK by phosphorylated EGFR also enables the activation of RAS or MEK in order to induce the expression of CCND1 (Zhang & Liu, 2002). Overall, these oncogenic pathways cross-regulate each other and are regulated by EGFR, ERK, and

Akt phosphorylation, forming an important network that enables the promotion of tumour cells to evade senescence and the acceleration of cell immortalization, proliferation, and metastasis in tumourigenesis. Therefore, this study highlights the importance of this network as it may serve as a promising subject for therapeutic development efforts and their associated biological functions and networks along with other common oncogenic pathways in tumourigenesis. In other words, the suppressor involved in targeting this network, especially the EGFR, could be combined with cytotoxic chemotherapeutic drugs or radiation therapy in affecting a cancer patient's prognosis. Interestingly, this network analysis also revealed putative novel oncogenes, namely ISG15 and MMP13 involved in the network signalling that linked with cell death and survival, cellular movement, cellular development. These genes were prioritized for further evaluation and characterization in OSCC by quantification of mRNA and protein expression levels as their clinical impact in oral cancer is poorly understood and literature suggests a high probability of involvement in tumourigenesis. These genes were found to harbor in rare amplicons (chromosome 1p36.33 and 11q22.2), however, it has been hypothesized that even rare amplicons would be informative regarding genes crucial in tumor development as the expression may be altered by either copy number changes or other mechanisms by disrupting the upstream or downstream genes in different mechanisms that control a particular pathway (Snijders *et al.*, 2005). Therefore, in order to test this hypothesis whether ISG15 is a driver gene in OSCC (as MMP13 roles in oral tumorigenesis has been studied previously). Therefore, ISG15 was selected for further downstream analysis which is to determine its gene and protein expression in OSCC samples as well as its functional roles via *in vitro* study using OSCC cell line models based on its involvement in the oncogenic network (cell death and survival, cellular movement, cellular development).

5.3 Identifying and determining the mRNA and protein expression of candidate gene and their correlation with clinical and socio-demographic parameters and as survival of patients

5.3.1 Matrix Metallopeptidase 13 (MMP13)

To date, few studies have demonstrated the over-expression of MMP13 mRNA and protein expression among OSCCs and head and neck SCCs (Johansson *et al.*, 1997; Cazorla *et al.*, 1998; Etoh *et al.*, 2000; P *et al.*, 2001; Culhaci *et al.*, 2004; Dunne *et al.*, 2005; Chiang *et al.*, 2006; Luukkaa *et al.*, 2006; Makinen *et al.*, 2012), although their role in oral tumourigenesis remains elusive. CNAs are widely documented as among the major drivers in cancer cases essentially through the process of altering the gene transcription levels (Albertson, 2006). In this study, the amplification of chromosome 11q22.2 was identified in 4 out of 75 OSCC samples that were subjected to array CGH analysis. Further identification of this amplicon revealed a candidate oncogene namely MMP13 that is involved in tumourigenesis with its metastasis ability. The identification of the four samples enabled the determination of the role of this gene in oral tumourigenesis using an independent set of samples at mRNA and protein levels and provides a stronger conclusion for the discovery of biomarkers in cancers (Hewitt *et al.*, 2004).

Although there is a lack of significant association between mRNA expressions of MMP13 with clinico-pathologic parameters, the over-expression of MMP13 mRNA was associated with poor prognosis. A similar observation has been reported on esophageal SCC (Etoh *et al.*, 2000). The literature review shows that, to date, only two investigations have been conducted on the prognostic value of MMP13 in OSCCs. The first study, by (Chiang *et al.*, 2006), did not observe any association between over-expression of MMP13 and clinical outcome as well as poor survival while the second,

which mainly focused on oral tongue SCC, found a significant role for MMP13 as a prognostic marker (Makinen *et al.*, 2012). In this study, the over-expression of MMP13 protein showed a significant association with advanced stage and lymph node metastasis. This observation reveals the proteolytic activity of MMP13 in the degradation of the extracellular matrix and basement membrane which enhances tumour progression and invasion in oral tumourigenesis. To the best of our knowledge, no extensive study has been conducted on the relationship between MMP13 protein expression and lymph node metastasis in OSCC, and these findings provide further support that MMP13 is involved in OSCC invasion and metastasis. In addition, it shows an association with poor prognosis. However, when adjusted for lymph node status and tumour staging, MMP13 was not shown to be an independent prognostic factor. This implies that the significance of using MMP13 as a prognostic marker may be more pronounced after taking into account the patient's lymph node status and tumour stage.

Taken together, the over-expression of MMP13 has been identified as an independent prognostic marker for OSCC at the mRNA level. In addition, increased expressions of MMP13 protein in OEDs and OSCC as compared to normal oral mucosa, and its correlation with advanced stage and lymph node metastasis of OSCC provide further evidence of its role in the genesis and progression of OSCC. Further investigations on the interaction of MMP13 with other potential genes or environmental risk factors would shed light on the complex role of this gene in oral tumourigenesis.

In this study, MMP13 protein was found to be highly expressed in the epithelial cells of OSCCs as compared to normal oral mucosal epithelial cells. In addition, the increased expressions of MMP13 protein from the epithelial cells of normal mucosa as compared to OSCC reflect the important role of this gene in the progression to OSCC. These current results are consistent with previously reported evidence conducted on OEDs and OSCCs (Chiang *et al.*, 2006). Hence, MMP13 protein might be considered as a useful predictive marker for OEDs with a risk of malignant transformation. However, as the sample size of OEDs was too small to draw any strong conclusions, further investigations are warranted.

5.4 Investigating the functional roles of a putative cancer gene using RNAi technique in OSCC cell lines model

5.4.1 Interferon Stimulated Gene (ISG15)

In this study, the amplification of the ISG15 was identified in chromosome 1p36.33 in 4 out of 75 OSCC samples using array CGH, and its mRNA and protein expressions were significantly over-expressed in OSCC compared to non-cancer oral mucosa samples and OEDs. Further, the high level of ISG15 expressions was shown to associate with gender, LNM, and pathological staging as well as poor prognosis in OSCC. To the best of our knowledge, this is the first attempt to use siRNA treatment to silence the ISG15 gene and to further characterize its oncogenic role in oral tumorigenesis. Although Duex *et al.* (2011) had transfected siRNA that targeted against ISG15 in the tongue SCC, glioma, and Hela cell lines, only the Hela and Glioma cell lines that were treated with siRNA of ISG15 were reported as having inhibited cell proliferation and induction of apoptosis. There was no further data that demonstrated this finding in the tongue SCC cell line employed in their study for the oncogenic roles of this gene in oral tumourigenesis.

ISG15 is an interferon regulated gene induced as a primary response with regard to various microbial and cell stress stimuli and encodes a 15kDA interferon inducible ubiquitin-like protein (Andersen & Hassel, 2006). As an ubiquitin-like protein, ISG15 is conjugated to cellular proteins *via* an enzymatic pathway comprising the E1, E2, and E3 enzymes that are not different from the ubiquitination mechanism (Pickart, 2001; Jeon *et al.*, 2010; Zhang & Zhang, 2011). However, the post-translational modification by ISG15 does not result in a proteasome-dependent degradation of ISG15 conjugates protein (ISGylation) which is not seen in ubiquitination (Zhang & Zhang, 2011). In ISGylation mechanism, the conjugation of ISG15 with protein substrates provides a tag that either marks the labelled protein for degradation or modulates its function and, therefore, disrupts the ubiquitination and alters the function of cellular proteins in cancer (Jeon *et al.*, 2010; Zhang & Zhang, 2011).

Andersen and Hassel (2006) indicate that the appropriate unperturbed regulation of ISG15 gives rise to a tumour suppressive effect. However, the dys-regulated over-expression of ISG15 in tumour cells results in an oncogenic effect that enhances tumour progression and is correlated with the malignant phenotype in tumorigenesis. In this study, the over-expression of ISG15 in its mRNA level was observed using qPCR in OSCC samples. The IHC analysis displayed an over-expression of ISG15 protein in OEDs with increasing grades of severity to OSCC indicating the involvement of this gene in the transformation from OEDs to invasive OSCC. These findings in the current study supported the study by Sumino *et al.* (2013) who found similar results in both OEDs and OSCC. Thus, this gene can be used to improve the diagnosis and early detection of oral cancer. In this way, improved diagnosis and early detection of oral cancer can contribute to the administration of appropriate management approaches to patients in terms of surgery, radiotherapy, or chemotherapy. To date, this study is the

first to significantly demonstrate the association of ISG15 expression with LNM and pathological staging in OSCC. These findings were also reported in hepatocellular carcinoma, breast and bladder cancer (Andersen *et al.*, 2006; Bektas *et al.*, 2008; Li *et al.*, 2014a). Apart from that, the over-expression of this gene has been reported to be associated with poorer prognosis in several types of cancers such as bladder, breast, and prostate cancers (Andersen *et al.*, 2006; Bektas *et al.*, 2008; Kiessling *et al.*, 2009). This study also demonstrates that the over-expression of this gene is associated with poor prognosis as evidenced by the Kaplan-Meier analysis. These findings further strengthen the value of ISG15 as a prognostic marker in OSCC which is in agreement with Sumino *et al.* (2013). These results may be translated into the possibility of potentially using ISG15 as a prognostic biomarker in OSCC patients. This may assist the surgeons or oncology team in making decisions as to the need to be more or less radical in planning the treatment for the OSCC patients. For example, surgeons may modify treatment procedures in OSCC patients with low-expressed ISG15, which may predict better survival, to avoid disfigurement and morbidity that may arise from overtreatment. Another explanation of perceiving this gene as a poor prognostic marker in OSCC patients might be due to its adverse response towards chemotherapeutic agents such as paclitaxel and 5-fluorouracil in the therapy of OSCC which were also reported in ovarian carcinoma xenograft and esophageal SCC (Bani *et al.*, 2004; Matsumura *et al.*, 2005).

It is well documented that the ISG15 protein exists in both free (intracellular) and conjugated form within the cells (ISGylation) with different cellular functions in tumourigenesis (Andersen & Hassel, 2006). In this study, the Western blot analysis showed that the OSCC cell lines expressed only the free form of ISG15. This observation was identified in hepatocellular and bladder cancer where the over-expression of the free form of the ISG15 but not its conjugates plays an important role in tumourigenesis (Andersen *et al.*, 2006; Li *et al.*, 2014a). However, a contrasting finding was reported by Desai (2015) who showed that free ISG15 was down-regulated and had an anti-tumour function and its conjugate form had a pro-tumour function in breast cancer. Although the amplification and over-expression of ISG15 has been reported in oral cancer (Vincent-Chong *et al.*, 2012; Sumino *et al.*, 2013), the mechanism of this gene in oral tumourigenesis remains unclear and as such, further downstream analysis should be performed to clarify its role in this area.

Chi *et al.* (2009) identified the ISG15 protein as over-expressed in OSCC lesions at different subsites within the oral cavity mainly in the tongue, cheeks, and gums. They also demonstrated that IFN-beta treatment in the OC3 cheek SCC cell line induced ISG15 expression and enhanced cell migration ability. However, the migration ability caused by ISG15 remains unknown as it may be driven by the IFN-beta treatment. Therefore, in this study, three OSCC cell lines derived from the gums, cheeks, and tongue SCC were employed as OSCC in the *in vitro* model to investigate the functional role(s) of ISG15 in oral tumorigenesis.

In this study, a knockdown of ISG15 suppressed cell proliferation and suggested to induce apoptosis in oral tumourigenesis, indicating that it might promote tumour cell survival and tumour growth in OSCC cells. Furthermore, the knockdown of the ISG15 might induce apoptosis, which is associated with the modulation of caspases 3 and caspases 7 that are known apoptosis related proteins. However, this notion requires experimental verification *via* apoptosis assay assessed by Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining to discriminate apoptotic cells from live cells and necrotic cells. The inhibition of ISG15 also suppressed tumour cell proliferation *via* MTT assay and increased the doubling time of oral cancer cells. The plausible mechanism driving this phenomenon could be the interaction with CCND1 and EGFR obtained from the network analysis that identified in this current study. The inhibition of ISG15 may reduce the expression of CCND1 and induce cell cycle arrest (Musgrove *et al.*, 2011). Apart from that, the role of EGFR in promoting tumour cell proliferation and tumour survival *via* various oncogenic pathways in cancer is well documented (Mendelsohn & Baselga, 2006). Therefore, ISG15 may exert its anti-apoptotic function by directly inhibiting caspase activities and up-regulating proliferative proteins, thus contributing to oral tumorigenesis.

In tumourigenesis, the resistance of tumour cells to apoptosis is an important characteristic in cancer development (Igney & Krammer, 2002) and resolving this resistance in cancer cells is crucial as it can lead to improvements in tumour treatment responses. This study also suggests that the anti-apoptotic function of ISG15 induced resistance to anti-cancer treatment such as cisplatin. The recent review by da Silva *et al.* (2012) indicated that cisplatin is one of the most common chemotherapy drugs used for primary or adjuvant treatment for locally advanced OSCC. Studies also showed that cisplatin-based induction chemotherapy has been reported to improve 5-year survival

rates in HNSCC cancer patients with locally advanced disease (Merlano *et al.*, 1996; Adelstein *et al.*, 2002). In this study, the knockdown of ISG15 enhanced cisplatin-induced cell death in OSCC cells and was further supported by elevated levels of cleaved caspases 3 and caspases 7 in ISG15 siRNA OSCC cells after cisplatin treatment. However, the reduction of the cell viability is more obvious particularly in ORL-188 compared to ORL-48 and ORL-204. The mechanism that lead to this observation is unclear due to the ISG15 protein expression for all cell lines were in similar level (refer to western blot result, Figure 4.13). Therefore, the mechanism that caused this event requires further study for confirmation. Overall, these results suggested that ISG15 inhibits cisplatin-induced cell death and contributes to cisplatin resistance in OSCC cells. Our findings suggest that ISG15 can serve as a specific predictor for response to cisplatin in OSCC. Further, a therapy targeting ISG15 in combination with cisplatin may overcome apoptotic resistance and improve responses in OSCC.

Oral cancer metastasis is a complex process involving the detachment of cells from tumour tissue, regulation of cell motility and invasion, and proliferation and invasion through the lymphatic system or blood vessels (Noguti *et al.*, 2012). Understanding the molecular mechanisms that mediate tumour invasion and metastasis may facilitate the identification of novel therapeutic targets for the management of tumour dissemination. In this study, over-expression of ISG15 was significantly associated with lymph node metastasis and advanced stages of OSCC. These findings suggest that ISG15 may have a role in driving disease spread. With this in mind, the role of ISG15 in increasing migratory and invasion potential was examined with the wound healing assay, transwell migration and invasion assays. Through these assays, it was evident that the knockdown of ISG15 suppressed tumour cell migration and invasion in OSCC cells. Therefore, this

study showed that ISG15 could promote cell migration and invasion and is likely to be involved in mechanisms of disease spread, which is consistent with the association of its expression with lymph node metastases and advanced stages of OSCC. Although the mechanism by which ISG15 could drive metastasis is still unclear, ISG15 could contribute to this phenotype by disrupting the cytoskeletal architecture and stabilizing the protein by inhibiting the ubiquitin/26S proteasome pathway which could affect cell motility (Desai *et al.*, 2012).

CHAPTER 6: CONCLUSIONS

6.1 Summary and conclusion of study

The primary objective of the current research was to determine CNAs in OSCC using array CGH. The genome-wide profiling results revealed multiple genomic regions, including amplifications on 1p, 3q, 5p, 7p, 8q, 9q, 10p, 11q, and deletions on 3p and 8p. The most frequently amplified CNAs were located on chromosome 8q22.3-q23.1 whereas the most frequently deleted CNAs were located on chromosome 3p21.31. The study also has identified chromosomes 3q, 8q and 11q had the highest number of changes in copy number at different regions across the entire length of the chromosome. Amplification of chromosome 7p was significantly associated with tumour size and tumour staging whereas amplification of chromosome 8q, 11q and loss of chromosome 8p were found to be significantly associated with tumour staging. Greater amplifications of chromosome 9p show a significant relationship in OSCC patients with lymph node metastasis ($p < 0.05$). Amplification of chromosome 8q, 11q, 7p, 9p and deletion of chromosome 8p were significantly associated with poor prognosis ($p < 0.05$). Amplification of chromosome 11q, 7p, 9p and deletion of chromosome 8p remained as an independent prognostic marker after adjustment for selected socio-demographic (age, gender, and risk habits) and clinico-pathologic parameters (tumour subsite, lymph node status and tumour staging) were made for OSCC. Amplification of a combination of chromosomes 7p8q9p11q considered as a 'genetic signature' remained as an independent prognostic marker after adjustment for selected socio-demographic (age, gender, and risk habits) and clinico-pathological parameters (tumour subsite, lymph node status and tumour staging) of OSCC. More than 50% of the selected amplified genes validated in the OSCC samples employed in

the genome-wide profiling study. The qPCR copy number analysis showed a similar pattern of the genetic signature (chromosome 7p8q9p11q) and their respective amplified gene namely EGFR, LRP12, TPM2 and CCND1, grouping together as a four amplified genes 'genetic signature' were found to be an independent prognostic marker after adjustment for selected socio-demographic (age, gender and risk habits) and clinico-pathological parameters (tumour subsite, lymph node status and tumour staging) of OSCC.

The second objective of this study was to identify the pathways, biological annotations and networks implicated in OSCC pathogenesis. The top most significant signalling pathway identified was integrin-linked kinase signalling pathway. The biological annotations associated with the CNAs include cell death and survival, cellular function and maintenance, cellular development, cellular growth and proliferation, and cellular movement. The most significant network was linked to cell death and survival, cellular movement, cellular development. The top most significant network harbours 73 genes among which the major core genes CCND1, RELA, TP63 and EGFR were hub nodes in the network, and formed interconnected auto-regulatory and feed forward circuitry.

The third objective of this study was to identify and to determine the mRNA and protein expression of the selected candidate genes and their correlation with clinical and socio-demographic parameters as well as the survival of patients. In this study, the expression of MMP13 and ISG15 were validated in Malaysian oral cancer patients. To the best of our knowledge, this is the largest study to demonstrate mRNA and protein expression using OSCC samples. The mRNA and protein expression levels were confirmed to be over-expressed significantly in OSCC in comparison to the non-cancer

oral mucosa samples for both the genes. Statistical analysis of both genes was associated with lymph node metastasis and tumour staging. Kaplan-Meier analysis revealed that over-expression of MMP13 and ISG15 genes were associated with poor prognosis. However, multivariate Cox regression model analysis for the over-expression of MMP13 and ISG15 genes do not support these genes to be independent prognostic markers in OSCC.

The last objective was to investigate the functional roles of the putative cancer gene using RNAi technique in OSCC cell lines model. In this study, the gene expression of ISG15 was reduced using siRNA that targeted ISG15 in oral cancer cell lines. *In vitro* experiments showed that reduced expression of ISG15 by siRNA inhibited cancer cell proliferation, migration, invasion and was more resistant to cell death compared to the non-targeting control. Reduced expression of ISG15 also resulted in an increase in susceptibility to cisplatin-induced cell death, as indicated by the increase in Caspase3/7 activation. Taken together, ISG15 drives cell proliferation and migration suggesting it may be driver gene in oral tumorigenesis, thus could be a potential therapeutic target for OSCC patients. Further, over-expression of ISG15 could also be novel clinical and prognostic biomarkers in OSCC and the inhibition of this gene could be a potential therapeutic target for OSCC patients.

6.2 Study limitations and recommendations

A limitation in this current study was that the copy number alterations were not analyzed parallel to the gene expression level. This can be achieved by integrating the copy number alterations with gene expression data derived from a public database such as the recent TCGA consortium exome sequencing and RNA sequencing data to

identify specific target genes that altered the gene dosage caused by the chromosomal alterations. Secondly, this *in vitro* functional study demonstrated that reduced expression of ISG15 enabled suppression of tumour cell proliferation, migration, invasion and were more resistant to apoptosis in oral carcinogenesis. However, this study did not verify that the inhibition of ISG15 gene expression prevents tumour growth and metastasis in oral cancer *in vivo.*

In the future, the findings from the high-resolution array CGH can be validated using a larger number of samples on lower resolution array CGH. This is proposed to reduce cost and to improve the probability of detecting recurrent segmental copy number alteration and other mechanisms such as epigenetic changes and mutations at the sequence level, which can contribute to specific gene deregulation. The present study identified genes residing in recurrent chromosomal regions of oral cancer. Therefore, in order to obtain a comprehensive picture of oral cancer, future efforts can be made to combine the array CGH approaches with transcriptomic and proteomic technologies. This can be done by integrating the copy number data with public expression datasets such as the recent TCGA consortium exome sequencing and RNA sequencing data in order to identify specific target genes that altere the gene dosage caused by the chromosomal alterations.

In the current study, gene knockdown of ISG15 was applied to three cell lines namely ORL-48T, ORL-188T and ORL-204T. These three cell lines may not represent all the different subsite of OSCC cells, and therefore, the study of ISG15 ideally should be repeated with OSCC cell lines from other oral cavity subsites and in other types of human cancers.

Gene targeting therapy has been suggested as a novel therapeutic that are likely to improve the prognosis for advanced cancer patients. ISG15 gene is ideal for gene therapy research as it is found to be over-expressed in OSCC cancer cell but not in the non-cancer oral mucosa tissue. However further investigation on the knockdown effect of the gene and its application *in vivo* will facilitate the development of ISG15-targeted OSCC treatment for clinical application. Apart from that, the ISG15 protein is involved in various pathways and cell types, therefore, future studies on the signalling mechanism including the use of specific inhibitors or neutralizing antibodies to particular signalling molecules, to elucidate the ISG15-related genes and pathways would be essential to consolidate the ISG15 signalling mechanism.

On the other hand, ISG15 is a secretory protein that can be detected in the bloodstream of patients; its potential as a prognostic or diagnostic marker should be a point of interest for future OSCC cases, and for that reason the ISG15 level in different cohorts of patients, as well as healthy individuals, should be studied to provide a clear reference for the application of ISG15 as a marker for OSCC detection.

REFERENCES

Aaronson, S. A. (1991). Growth factors and cancer. *Science, 254*(5035), 1146-1153.

Abbott, R. G., Forrest, S., & Pienta, K. J. (2006). Simulating the hallmarks of cancer. *Artif Life, 12*(4), 617-634.

Adelstein, D. J., Saxton, J. P., Lavertu, P., Rybicki, L. A., Esclamado, R. M., Wood, B. G., Strome, M., & Carroll, M. A. (2002). Maximizing local control and organ preservation in stage IV squamous cell head and neck cancer With hyperfractionated radiation and concurrent chemotherapy. *J Clin Oncol, 20*(5), 1405-1410.

Agrawal, N., Frederick, M. J., Pickering, C. R., Bettegowda, C., Chang, K., Li, R. J., Fakhry, C., Xie, T. X., Zhang, J., Wang, J., Zhang, N., El-Naggar, A. K., Jasser, S. A., Weinstein, J. N., Trevino, L., Drummond, J. A., Muzny, D. M., Wu, Y., Wood, L. D., Hruban, R. H., Westra, W. H., Koch, W. M., Califano, J. A., Gibbs, R. A., Sidransky, D., Vogelstein, B., Velculescu, V. E., Papadopoulos, N., Wheeler, D. A., Kinzler, K. W., & Myers, J. N. (2011). Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science, 333*(6046), 1154-1157.

Akervall, J. A., Jin, Y., Wennerberg, J. P., Zatterstrom, U. K., Kjellen, E., Mertens, F., Willen, R., Mandahl, N., Heim, S., & Mitelman, F. (1995). Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. *Cancer, 76*(5), 853-859.

Albertson, D. G. (2006). Gene amplification in cancer. *Trends Genet, 22*(8), 447-455.

Albertson, D. G., Collins, C., McCormick, F., & Gray, J. W. (2003). Chromosome aberrations in solid tumors. *Nat Genet, 34*(4), 369-376.

Almangush, A., Bello, I. O., Keski-Santti, H., Makinen, L. K., Kauppila, J. H., Pukkila, M., Hagstrom, J., Laranne, J., Tommola, S., Nieminen, O., Soini, Y., Kosma, V. M., Koivunen, P., Grenman, R., Leivo, I., & Salo, T. (2014). Depth of invasion, tumor budding, and worst pattern of invasion: prognostic indicators in early-stage oral tongue cancer. *Head Neck, 36*(6), 811-818.

Ambatipudi, S., Gerstung, M., Gowda, R., Pai, P., Borges, A. M., Schaffer, A. A., Beerenwinkel, N., & Mahimkar, M. B. (2011). Genomic profiling of advanced-stage oral cancers reveals chromosome 11q alterations as markers of poor clinical outcome. *PLoS One, 6*(2), e17250.

Amtha, R., Ching, C. S., Zain, R., Razak, I. A., Basuki, B., Roeslan, B. O., Gautama, W., & Purwanto, D. (2009). GSTM1, GSTT1 and CYP1A1 polymorphisms and risk of oral cancer: a case-control study in Jakarta, Indonesia. *Asian Pac J Cancer Prev, 10*(1), 21-26.

Andersen, J. B., Aaboe, M., Borden, E. C., Goloubeva, O. G., Hassel, B. A., & Orntoft, T. F. (2006). Stage-associated overexpression of the ubiquitin-like protein, ISG15, in bladder cancer. *Br J Cancer, 94*(10), 1465-1471.

Andersen, J. B., & Hassel, B. A. (2006). The interferon regulated ubiquitin-like protein, ISG15, in tumorigenesis: friend or foe? *Cytokine Growth Factor Rev, 17*(6), 411-421.

Anneroth, G., Batsakis, J., & Luna, M. (1987). Review of the literature and a recommended system of malignancy grading in oral squamous cell carcinomas. *Scand J Dent Res, 95*(3), 229-249.

Arduino, P. G., Carrozzo, M., Chiecchio, A., Broccoletti, R., Tirone, F., Borra, E., Bertolusso, G., & Gandolfo, S. (2008). Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: a retrospective study of 334 cases. *J Oral Maxillofac Surg, 66*(8), 1570-1579.

Argiris, A., Karamouzis, M. V., Raben, D., & Ferris, R. L. (2008). Head and neck cancer. *Lancet, 371*(9625), 1695-1709.

Arthur, K., & Farr, H. W. (1972). Prognostic significance of histologic grade in epidermoid carcinoma of the mouth and pharynx. *Am J Surg, 124*(4), 489-492.

Asai, T., Tomita, Y., Nakatsuka, S., Hoshida, Y., Myoui, A., Yoshikawa, H., & Aozasa, K. (2002). VCP (p97) regulates NFkappaB signaling pathway, which is important for metastasis of osteosarcoma cell line. *Jpn J Cancer Res, 93*(3), 296-304.

Baak, J. P., Path, F. R., Hermsen, M. A., Meijer, G., Schmidt, J., & Janssen, E. A. (2003). Genomics and proteomics in cancer. *Eur J Cancer, 39*(9), 1199-1215.

Bagan, J., Sarrion, G., & Jimenez, Y. (2010). Oral cancer: clinical features. *Oral Oncol, 46*(6), 414-417.

Bani, M. R., Nicoletti, M. I., Alkharouf, N. W., Ghilardi, C., Petersen, D., Erba, E., Sausville, E. A., Liu, E. T., & Giavazzi, R. (2004). Gene expression correlating with response to paclitaxel in ovarian carcinoma xenografts. *Mol Cancer Ther, 3*(2), 111-121.

Bankfalvi, A., & Piffko, J. (2000). Prognostic and predictive factors in oral cancer: the role of the invasive tumour front. *J Oral Pathol Med, 29*(7), 291-298.

Banoczy, J., Gintner, Z., & Dombi, C. (2001). Tobacco use and oral leukoplakia. *J Dent Educ, 65*(4), 322-327.

Bazley, L. A., & Gullick, W. J. (2005). The epidermal growth factor receptor family. *Endocr Relat Cancer, 12 Suppl 1*, S17-27.

Beckman, R. A., & Loeb, L. A. (2005). Genetic instability in cancer: theory and experiment. *Semin Cancer Biol, 15*(6), 423-435.

Bejjani, B. A., & Shaffer, L. G. (2006). Application of array-based comparative genomic hybridization to clinical diagnostics. *J Mol Diagn, 8*(5), 528-533.

Bektas, N., Noetzel, E., Veeck, J., Press, M. F., Kristiansen, G., Naami, A., Hartmann, A., Dimmler, A., Beckmann, M. W., Knuchel, R., Fasching, P. A., & Dahl, E. (2008). The ubiquitin-like molecule interferon-stimulated gene 15 (ISG15) is a potential prognostic marker in human breast cancer. *Breast Cancer Res, 10*(4), R58.

Berke, J. D., Sgambato, V., Zhu, P. P., Lavoie, B., Vincent, M., Krause, M., & Hyman, S. E. (2001). Dopamine and glutamate induce distinct striatal splice forms of Ania-6, an RNA polymerase II-associated cyclin. *Neuron, 32*(2), 277-287.

Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefebvre, J. L., Greiner, R. H., Giralt, J., Maingon, P., Rolland, F., Bolla, M., Cognetti, F., Bourhis, J., Kirkpatrick, A., van Glabbeke, M., European Organization for, R., & Treatment of Cancer, T. (2004). Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med, 350*(19), 1945-1952.

Beroukhim, R., Mermel, C. H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J. S., Dobson, J., Urashima, M., Mc Henry, K. T., Pinchback, R. M., Ligon, A. H., Cho, Y. J., Haery, L., Greulich, H., Reich, M., Winckler, W., Lawrence, M. S., Weir, B. A., Tanaka, K. E., Chiang, D. Y., Bass, A. J., Loo, A., Hoffman, C., Prensner, J., Liefeld, T., Gao, Q., Yecies, D., Signoretti, S., Maher, E., Kaye, F. J., Sasaki, H., Tepper, J. E., Fletcher, J. A., Tabernero, J., Baselga, J., Tsao, M. S., Demichelis, F., Rubin, M. A., Janne, P. A., Daly, M. J., Nucera, C., Levine, R. L., Ebert, B. L., Gabriel, S., Rustgi, A. K., Antonescu, C. R., Ladanyi, M., Letai, A., Garraway, L. A., Loda, M., Beer, D. G., True, L. D., Okamoto, A., Pomeroy, S. L., Singer, S., Golub, T. R., Lander, E. S., Getz, G., Sellers, W. R., & Meyerson, M. (2010). The landscape of somatic copy-number alteration across human cancers. *Nature, 463*(7283), 899-905.

Bertrand, J. R., Pottier, M., Vekris, A., Opolon, P., Maksimenko, A., & Malvy, C. (2002). Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem Biophys Res Commun, 296*(4), 1000-1004.

Bitu, C. C., Destro, M. F., Carrera, M., da Silva, S. D., Graner, E., Kowalski, L. P., Soares, F. A., & Coletta, R. D. (2012). HOXA1 is overexpressed in oral squamous cell carcinomas and its expression is correlated with poor prognosis. *BMC Cancer, 12*, 146.

Blanchard, P., Baujat, B., Holostenco, V., Bourredjem, A., Baey, C., Bourhis, J., Pignon, J. P., & group, M.-C. C. (2011). Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): a comprehensive analysis by tumour site. *Radiother Oncol, 100*(1), 33-40.

Bockmuhl, U., Schluns, K., Kuchler, I., Petersen, S., & Petersen, I. (2000). Genetic imbalances with impact on survival in head and neck cancer patients. *Am J Pathol, 157*(2), 369-375.

British, D. A. (2000). Opportunistic oral cancer screening. *BDA Occasional Paper, 6*(1), 1-36.

Brockton, N. T., Klimowicz, A. C., Bose, P., Petrillo, S. K., Konno, M., Rudmik, L., Dean, M., Nakoneshny, S. C., Matthews, T. W., Chandarana, S., Lau, H. Y., Magliocco, A. M., & Dort, J. C. (2012). High stromal carbonic anhydrase IX expression is associated with nodal metastasis and decreased survival in patients with surgically-treated oral cavity squamous cell carcinoma. *Oral Oncol, 48*(7), 615-622.

Bronstad, I., Wolff, A. S., Lovas, K., Knappskog, P. M., & Husebye, E. S. (2011). Genome-wide copy number variation (CNV) in patients with autoimmune Addison's disease. *BMC Med Genet, 12*, 111.

Broumand, V., Lozano, T. E., & Gomez, J. A. (2006). Evaluation and staging of oral cancer. *Oral Maxillofac Surg Clin North Am, 18*(4), 435-444.

Callender, T., el-Naggar, A. K., Lee, M. S., Frankenthaler, R., Luna, M. A., & Batsakis, J. G. (1994). PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer, 74*(1), 152-158.

Cazorla, M., Hernandez, L., Nadal, A., Balbin, M., Lopez, J. M., Vizoso, F., Fernandez, P. L., Iwata, K., Cardesa, A., Lopez-Otin, C., & Campo, E. (1998). Collagenase-3 expression is associated with advanced local invasion in human squamous cell carcinomas of the larynx. *J Pathol, 186*(2), 144-150.

Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer, 2*(8), 563-572.

Chang, Y. C., Nieh, S., Chen, S. F., Jao, S. W., Lin, Y. L., & Fu, E. (2010). Invasive pattern grading score designed as an independent prognostic indicator in oral squamous cell carcinoma. *Histopathology, 57*(2), 295-303.

Chauhan, S. C., Ebeling, M. C., Maher, D. M., Koch, M. D., Watanabe, A., Aburatani, H., Lio, Y., & Jaggi, M. (2012). MUC13 mucin augments pancreatic tumorigenesis. *Mol Cancer Ther, 11*(1), 24-33.

Chauhan, S. C., Vannatta, K., Ebeling, M. C., Vinayek, N., Watanabe, A., Pandey, K. K., Bell, M. C., Koch, M. D., Aburatani, H., Lio, Y., & Jaggi, M. (2009). Expression and functions of transmembrane mucin MUC13 in ovarian cancer. *Cancer Res, 69*(3), 765-774.

Chen, C., Zhang, Y., Loomis, M. M., Upton, M. P., Lohavanichbutr, P., Houck, J. R., Doody, D. R., Mendez, E., Futran, N., Schwartz, S. M., & Wang, P. (2015). Genome-Wide Loss of Heterozygosity and DNA Copy Number Aberration in HPV-Negative Oral Squamous Cell Carcinoma and Their Associations with Disease-Specific Survival. *PLoS One, 10*(8), e0135074.

Chen, G., Bhojani, M. S., Heaford, A. C., Chang, D. C., Laxman, B., Thomas, D. G., Griffin, L. B., Yu, J., Coppola, J. M., Giordano, T. J., Lin, L., Adams, D., Orringer, M. B., Ross, B. D., Beer, D. G., & Rehemtulla, A. (2005). Phosphorylated FADD induces NF-kappaB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. *Proc Natl Acad Sci U S A, 102*(35), 12507-12512.

Chen, I. H., Chang, J. T., Liao, C. T., Wang, H. M., Hsieh, L. L., & Cheng, A. J. (2003). Prognostic significance of EGFR and Her-2 in oral cavity cancer in betel quid prevalent area cancer prognosis. *Br J Cancer, 89*(4), 681-686.

Chen, Y. J., Lin, S. C., Kao, T., Chang, C. S., Hong, P. S., Shieh, T. M., & Chang, K. W. (2004). Genome-wide profiling of oral squamous cell carcinoma. *J Pathol, 204*(3), 326-332.

Chi, L. M., Lee, C. W., Chang, K. P., Hao, S. P., Lee, H. M., Liang, Y., Hsueh, C., Yu, C. J., Lee, I. N., Chang, Y. J., Lee, S. Y., Yeh, Y. M., Chang, Y. S., Chien, K. Y., & Yu, J. S. (2009). Enhanced interferon signaling pathway in oral cancer revealed by quantitative proteome analysis of microdissected specimens using 16O/18O labeling and integrated two-dimensional LC-ESI-MALDI tandem MS. *Mol Cell Proteomics, 8*(7), 1453-1474.

Chiang, W. C., Wong, Y. K., Lin, S. C., Chang, K. W., & Liu, C. J. (2006). Increase of MMP-13 expression in multi-stage oral carcinogenesis and epigallocatechin-3-gallate suppress MMP-13 expression. *Oral Dis, 12*(1), 27-33.

Choi, S., & Myers, J. N. (2008). Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *J Dent Res, 87*(1), 14-32.

Ciardiello, F., De Vita, F., Orditura, M., & Tortora, G. (2004). The role of EGFR inhibitors in nonsmall cell lung cancer. *Curr Opin Oncol, 16*(2), 130-135.

Coleman, W. B., & Tsongalis, G. J. (2002). The role of genomic instability in the development of human cancer. In W. B. Coleman & G. J. Tsongalis (Eds.), *The Molecular Basis of Human Cancer* (pp. 115-142). New York Springer Science+Business Media

Cooper, G. M. (2000). Tumor Suppressor Genes *The Cell: A Molecular Approach.* Washington: ASM Press.

Cooper, J. S., Pajak, T. F., Forastiere, A. A., Jacobs, J., Campbell, B. H., Saxman, S. B., Kish, J. A., Kim, H. E., Cmelak, A. J., Rotman, M., Machtay, M., Ensley, J. F., Chao, K. S., Schultz, C. J., Lee, N., Fu, K. K., & Radiation Therapy Oncology Group, I. (2004). Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med, 350*(19), 1937-1944.

Cordes, N., & van Beuningen, D. (2003). Cell adhesion to the extracellular matrix protein fibronectin modulates radiation-dependent G2 phase arrest involving integrin-linked kinase (ILK) and glycogen synthase kinase-3beta (GSK-3beta) in vitro. *Br J Cancer, 88*(9), 1470-1479.

Croce, C. M. (2008). Oncogenes and cancer. *N Engl J Med, 358*(5), 502-511.

Croce, C. M., Sozzi, G., & Huebner, K. (1999). Role of FHIT in human cancer. *J Clin Oncol, 17*(5), 1618-1624.

Culhaci, N., Metin, K., Copcu, E., & Dikicioglu, E. (2004). Elevated expression of MMP-13 and TIMP-1 in head and neck squamous cell carcinomas may reflect increased tumor invasiveness. *BMC Cancer, 4*, 42.

da Silva, S. D., Alaoui-Jamali, M. A., Soares, F. A., Carraro, D. M., Brentani, H. P., Hier, M., Rogatto, S. R., & Kowalski, L. P. (2014). TWIST1 is a molecular marker for a poor prognosis in oral cancer and represents a potential therapeutic target. *Cancer, 120*(3), 352-362.

da Silva, S. D., Hier, M., Mlynarek, A., Kowalski, L. P., & Alaoui-Jamali, M. A. (2012). Recurrent oral cancer: current and emerging therapeutic approaches. *Front Pharmacol, 3*, 149.

Das, R. K., Pal, M., Barui, A., Paul, R. R., Chakraborty, C., Ray, A. K., Sengupta, S., & Chatterjee, J. (2010). Assessment of malignant potential of oral submucous fibrosis through evaluation of p63, E-cadherin and CD105 expression. *J Clin Pathol, 63*(10), 894-899.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab, 7*(1), 11-20.

Deheuninck, J., & Luo, K. (2009). Ski and SnoN, potent negative regulators of TGF-beta signaling. *Cell Res, 19*(1), 47-57.

Denhart, B. C., Guidi, A. J., Tognazzi, K., Dvorak, H. F., & Brown, L. F. (1997). Vascular permeability factor/vascular endothelial growth factor and its receptors in oral and laryngeal squamous cell carcinoma and dysplasia. *Lab Invest, 77*(6), 659-664.

Desai, S. D. (2015). ISG15: A double edged sword in cancer. *Oncoimmunology, 4*(12), e1052935.

Desai, S. D., Reed, R. E., Burks, J., Wood, L. M., Pullikuth, A. K., Haas, A. L., Liu, L. F., Breslin, J. W., Meiners, S., & Sankar, S. (2012). ISG15 disrupts cytoskeletal architecture and promotes motility in human breast cancer cells. *Exp Biol Med (Maywood), 237*(1), 38-49.

Dienstmann, R., Jang, I. S., Bot, B., Friend, S., & Guinney, J. (2015). Database of genomic biomarkers for cancer drugs and clinical targetability in solid tumors. *Cancer Discov, 5*(2), 118-123.

Dube, S., Thomas, A., Abbott, L., Benz, P., Mitschow, C., Dube, D. K., & Poiesz, B. J. (2016). Expression of tropomyosin 2 gene isoforms in human breast cancer cell lines. *Oncol Rep, 35*(6), 3143-3150.

Duex, J. E., Comeau, L., Sorkin, A., Purow, B., & Kefas, B. (2011). Usp18 regulates epidermal growth factor (EGF) receptor expression and cancer cell survival via microRNA-7. *J Biol Chem, 286*(28), 25377-25386.

Dunne, A. A., Mandic, R., Falkenberg, S., Dalchow, C. V., Sesterhenn, A. M., & Werner, J. A. (2005). RT-PCR expression profiling of matrix metalloproteinases and their specific inhibitors in cell lines and fresh biopsies of squamous cell carcinomas of the head and neck. *In Vivo, 19*(5), 943-948.

El-Naaj, I. A., Leiser, Y., Shveis, M., Sabo, E., & Peled, M. (2011). Incidence of oral cancer occult metastasis and survival of T1-T2N0 oral cancer patients. *J Oral Maxillofac Surg, 69*(10), 2674-2679.

Elmore, S. A. (2006). Histopathology of the lymph nodes. *Toxicol Pathol, 34*(5), 425-454.

Etoh, T., Inoue, H., Yoshikawa, Y., Barnard, G. F., Kitano, S., & Mori, M. (2000). Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness. *Gut, 47*(1), 50-56.

Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature, 411*(6835), 342-348.

Eversole, L. R. (2001). *Clinical outline of oral pathology: diagnosis and treatment*. Shelton: People's Medical Publishing House.

Farber, E. (1984). The multistep nature of cancer development. *Cancer Res, 44*(10), 4217-4223.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D., & Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer, 136*(5), E359-386.

Field, J. K. (1995). The role of oncogenes and tumour-suppressor genes in the aetiology of oral, head and neck squamous cell carcinoma. *J R Soc Med, 88*(1), 35P-39P.

Folkman, J. (1990). What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst, 82*(1), 4-6.

Folkman, J. (2006). Angiogenesis. *Annu Rev Med, 57*, 1-18.

Fröhling, S., & Döhner, H. (2008). Chromosomal abnormalities in cancer. *New England Journal of Medicine, 359*(7), 722-734.

Fujita, M., Hirokawa, Y., Kashiwado, K., Akagi, Y., Kashimoto, K., Kiriu, H., Matsuura, K., & Ito, K. (1999). Interstitial brachytherapy for stage I and II squamous cell carcinoma of the oral tongue: factors influencing local control and soft tissue complications. *Int J Radiat Oncol Biol Phys, 44*(4), 767-775.

Furness, S., Glenny, A. M., Worthington, H. V., Pavitt, S., Oliver, R., Clarkson, J. E., Macluskey, M., Chan, K. K., & Conway, D. I. (2011). Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. *Cochrane Database Syst Rev*(4), CD006386.

Gandini, S., Negri, E., Boffetta, P., La Vecchia, C., & Boyle, P. (2012). Mouthwash and oral cancer risk quantitative meta-analysis of epidemiologic studies. *Ann Agric Environ Med, 19*(2), 173-180.

Garewal, H. (1995). Antioxidants in oral cancer prevention. *Am J Clin Nutr, 62*(6 Suppl), 1410S-1416S.

Garnis, C., Campbell, J., Zhang, L., Rosin, M. P., & Lam, W. L. (2004a). OCGR array: an oral cancer genomic regional array for comparative genomic hybridization analysis. *Oral oncology, 40*(5), 511-519.

Garnis, C., Chari, R., Buys, T. P., Zhang, L., Ng, R. T., Rosin, M. P., & Lam, W. L. (2009). Genomic imbalances in precancerous tissues signal oral cancer risk. *Mol Cancer, 8*, 50.

Garnis, C., Coe, B. P., Zhang, L., Rosin, M. P., & Lam, W. L. (2004b). Overexpression of LRP12, a gene contained within an 8q22 amplicon identified by high-resolution array CGH analysis of oral squamous cell carcinomas. *Oncogene, 23*(14), 2582-2586.

Gisselsson, D., Jonson, T., Yu, C., Martins, C., Mandahl, N., Wiegant, J., Jin, Y., Mertens, F., & Jin, C. (2002). Centrosomal abnormalities, multipolar mitoses, and chromosomal instability in head and neck tumours with dysfunctional telomeres. *Br J Cancer, 87*(2), 202-207.

Gollin, S. M. (2005). Mechanisms leading to chromosomal instability. *Semin Cancer Biol, 15*(1), 33-42.

Gordon, K. E., Ireland, H., Roberts, M., Steeghs, K., McCaul, J. A., MacDonald, D. G., & Parkinson, E. K. (2003). High levels of telomere dysfunction bestow a selective disadvantage during the progression of human oral squamous cell carcinoma. *Cancer Res, 63*(2), 458-467.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell, 84*(3), 345-357.

Gupta, B., & Johnson, N. W. (2014). Systematic review and meta-analysis of association of smokeless tobacco and of betel quid without tobacco with incidence of oral cancer in South Asia and the Pacific. *PLoS One, 9*(11), e113385.

Haber, D. A., & Settleman, J. (2007). Cancer: drivers and passengers. *Nature, 446*(7132), 145-146.

Hahn, M., Hagedorn, G., Kuhlisch, E., Schackert, H. K., & Eckelt, U. (2002). Genetic polymorphisms of drug-metabolizing enzymes and susceptibility to oral cavity cancer. *Oral Oncol, 38*(5), 486-490.

Hamakawa, H., Nakashiro, K., Sumida, T., Shintani, S., Myers, J. N., Takes, R. P., Rinaldo, A., & Ferlito, A. (2008). Basic evidence of molecular targeted therapy for oral cancer and salivary gland cancer. *Head Neck, 30*(6), 800-809.

Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell, 86*(3), 353-364.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hayflick, L. (1997). Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc), 62*(11), 1180-1190.

He, L. H., Ma, Q., Shi, Y. H., Ge, J., Zhao, H. M., Li, S. F., & Tong, Z. S. (2013). CHL1 is involved in human breast tumorigenesis and progression. *Biochem Biophys Res Commun, 438*(2), 433-438.

Helen-Ng, L. C., Razak, I. A., Ghani, W. M., Marhazlinda, J., Norain, A. T., Raja Jallaludin, R. L., Rahman, Z. A., Abdullah, N., & Zain, R. B. (2012). Dietary pattern and oral cancer risk--a factor analysis study. *Community Dent Oral Epidemiol, 40*(6), 560-566.

Herbst, R. S. (2004). Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys, 59*(2 Suppl), 21-26.

Herity, B., Moriarty, M., Bourke, G. J., & Daly, L. (1981). A case-control study of head and neck cancer in the Republic of Ireland. *Br J Cancer, 43*(2), 177-182.

Herrero, R., Castellsague, X., Pawlita, M., Lissowska, J., Kee, F., Balaram, P., Rajkumar, T., Sridhar, H., Rose, B., Pintos, J., Fernandez, L., Idris, A., Sanchez, M. J., Nieto, A., Talamini, R., Tavani, A., Bosch, F. X., Reidel, U., Snijders, P. J., Meijer, C. J., Viscidi, R., Munoz, N., Franceschi, S., & Group, I. M. O. C. S. (2003). Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *J Natl Cancer Inst, 95*(23), 1772-1783.

Heselmeyer, K., Schrock, E., du Manoir, S., Blegen, H., Shah, K., Steinbeck, R., Auer, G., & Ried, T. (1996). Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc Natl Acad Sci U S A, 93*(1), 479-484.

Hewitt, S. M., Dear, J., & Star, R. A. (2004). Discovery of protein biomarkers for renal diseases. *J Am Soc Nephrol, 15*(7), 1677-1689.

Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A., & Weinberg, R. A. (1994). Function of a human cyclin gene as an oncogene. *Proc Natl Acad Sci U S A, 91*(2), 709-713.

Horner, M., Ries, L., Krapcho, M., Neyman, N., Aminou, R., Howlader, N., Altekruse, S., Feuer, E., Huang, L., & Mariotto, A. (2009). SEER Cancer Statistics Review, 1975-2006, National Cancer Institute. Bethesda, MD. https://seer.cancer.gov/archive/csr/1975_2006/

Howlader, N., Ries, L. A., Mariotto, A. B., Reichman, M. E., Ruhl, J., & Cronin, K. A. (2010). Improved estimates of cancer-specific survival rates from population-based data. *J Natl Cancer Inst, 102*(20), 1584-1598.

Hsieh, L. L., Wang, P. F., Chen, I. H., Liao, C. T., Wang, H. M., Chen, M. C., Chang, J. T., & Cheng, A. J. (2001). Characteristics of mutations in the p53 gene in oral squamous cell carcinoma associated with betel quid chewing and cigarette smoking in Taiwanese. *Carcinogenesis, 22*(9), 1497-1503.

Huang, X., Godfrey, T. E., Gooding, W. E., McCarty, K. S., Jr., & Gollin, S. M. (2006). Comprehensive genome and transcriptome analysis of the 11q13 amplicon in human oral cancer and synteny to the 7F5 amplicon in murine oral carcinoma. *Genes Chromosomes Cancer, 45*(11), 1058-1069.

Hunter, T., & Pines, J. (1991). Cyclins and cancer. *Cell, 66*(6), 1071-1074.

Hunter, T., & Pines, J. (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell, 79*(4), 573-582.

IARC. (2004a). Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. *IARC Monogr Eval Carcinog Risks Hum, 85*, 1-334.

IARC. (2004b). Tobacco smoke and involuntary smoking. *IARC Monogr Eval Carcinog Risks Hum, 83*, 1-1438.

IARC. (2007). Smokeless tobacco and some tobacco-specific N-nitrosamines. *IARC Monogr Eval Carcinog Risks Hum, 89*, 1-592.

IARC. (2010). Alcohol consumption and ethyl carbamate. *IARC Monogr Eval Carcinog Risks Hum, 96*, 3-1383.

ICGC. (2013). Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups. *Nat Commun, 4*, 2873.

Igney, F. H., & Krammer, P. H. (2002). Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer, 2*(4), 277-288.

Inazawa, J., Inoue, J., & Imoto, I. (2004). Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. *Cancer Sci, 95*(7), 559-563.

International Human Genome Sequencing, C. (2004). Finishing the euchromatic sequence of the human genome. *Nature, 431*(7011), 931-945.

Ishitoya, J., Toriyama, M., Oguchi, N., Kitamura, K., Ohshima, M., Asano, K., & Yamamoto, T. (1989). Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck. *Br J Cancer, 59*(4), 559-562.

Ivanov, S. V., Kuzmin, I., Wei, M. H., Pack, S., Geil, L., Johnson, B. E., Stanbridge, E. J., & Lerman, M. I. (1998). Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc Natl Acad Sci U S A, 95*(21), 12596-12601.

Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J. C., Trent, J. M., Staudt, L. M., Hudson, J., Jr., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., & Brown, P. O. (1999). The transcriptional program in the response of human fibroblasts to serum. *Science, 283*(5398), 83-87.

Jeon, Y. J., Yoo, H. M., & Chung, C. H. (2010). ISG15 and immune diseases. *Biochim Biophys Acta, 1802*(5), 485-496.

Jiang, L., Zeng, X., Yang, H., Wang, Z., Shen, J., Bai, J., Zhang, Y., Gao, F., Zhou, M., & Chen, Q. (2008). Oral cancer overexpressed 1 (ORAOV1): a regulator for the cell growth and tumor angiogenesis in oral squamous cell carcinoma. *Int J Cancer, 123*(8), 1779-1786.

Johansson, N., Airola, K., Grenman, R., Kariniemi, A. L., Saarialho-Kere, U., & Kahari, V. M. (1997). Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am J Pathol, 151*(2), 499-508.

Johnson, N. W. (1991). Orofacial neoplasms: global epidemiology, risk factors and recommendations for research. *Int Dent J, 41*(6), 365-375.

Johnson , N. W. (2003). Global epidemiology. In J. N. W. Shah J. P., Batsakis J. G (Ed.), *Oral cancer* (pp. 3-4). New York: Martin Dunitz.

Jones, R. G., & Thompson, C. B. (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. *Genes Dev, 23*(5), 537-548.

Jourenkova-Mironova, N., Voho, A., Bouchardy, C., Wikman, H., Dayer, P., Benhamou, S., & Hirvonen, A. (1999). Glutathione S-transferase GSTM1, GSTM3, GSTP1 and GSTT1 genotypes and the risk of smoking-related oral and pharyngeal cancers. *Int J Cancer, 81*(1), 44-48.

Kallioniemi, A. (2008). CGH microarrays and cancer. *Curr Opin Biotechnol, 19*(1), 36-40.

Kalyankrishna, S., & Grandis, J. R. (2006). Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol, 24*(17), 2666-2672.

Kannan, S., Tahara, H., Yokozaki, H., Mathew, B., Nalinakumari, K. R., Nair, M. K., & Tahara, E. (1997). Telomerase activity in premalignant and malignant lesions of human oral mucosa. *Cancer Epidemiol Biomarkers Prev, 6*(6), 413-420.

Katayama, H., Brinkley, W. R., & Sen, S. (2003). The Aurora kinases: role in cell transformation and tumorigenesis. *Cancer Metastasis Rev, 22*(4), 451-464.

Katoh, T., Kaneko, S., Kohshi, K., Munaka, M., Kitagawa, K., Kunugita, N., Ikemura, K., & Kawamoto, T. (1999). Genetic polymorphisms of tobacco- and alcohol-related metabolizing enzymes and oral cavity cancer. *Int J Cancer, 83*(5), 606-609.

Kiessling, A., Hogrefe, C., Erb, S., Bobach, C., Fuessel, S., Wessjohann, L., & Seliger, B. (2009). Expression, regulation and function of the ISGylation system in prostate cancer. *Oncogene, 28*(28), 2606-2620.

Kietthubthew, S., Sriplung, H., & Au, W. W. (2001). Genetic and environmental interactions on oral cancer in Southern Thailand. *Environ Mol Mutagen, 37*(2), 111-116.

Kinzler, K. W., & Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature, 386*(6627), 761, 763.

Kinzler, K. W., & Vogelstein, B. (1998). Landscaping the cancer terrain. *Science, 280*(5366), 1036-1037.

Kirkitadze, M. D., & Barlow, P. N. (2001). Structure and flexibility of the multiple domain proteins that regulate complement activation. *Immunol Rev, 180*, 146-161.

Kleihues, P., & Stewart, B. W. (2003). World cancer report.

Knudson, A. G. (2001). Two genetic hits (more or less) to cancer. *Nat Rev Cancer, 1*(2), 157-162.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A, 68*(4), 820-823.

Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., & Kallioniemi, O. P. (1998). Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med, 4*(7), 844-847.

Koontongkaew, S., Chareonkitkajorn, L., Chanvitan, A., Leelakriangsak, M., & Amornphimoltham, P. (2000). Alterations of p53, pRb, cyclin D(1) and cdk4 in human oral and pharyngeal squamous cell carcinomas. *Oral Oncol, 36*(4), 334-339.

Kowalski, L. P., Bagietto, R., Lara, J. R., Santos, R. L., Tagawa, E. K., & Santos, I. R. (1999). Factors influencing contralateral lymph node metastasis from oral carcinoma. *Head Neck, 21*(2), 104-110.

Kowalski, L. P., Hashimoto, I., & Magrin, J. (1993). End results of 114 extended "commando" operations for retromolar trigone carcinoma. *Am J Surg, 166*(4), 374-379.

Kozomara, R., Jovic, N., Magic, Z., Brankovic-Magic, M., & Minic, V. (2005). p53 mutations and human papillomavirus infection in oral squamous cell carcinomas: correlation with overall survival. *J Craniomaxillofac Surg, 33*(5), 342-348.

Kujan, O., Khattab, A., Oliver, R. J., Roberts, S. A., Thakker, N., & Sloan, P. (2007). Why oral histopathology suffers inter-observer variability on grading oral epithelial dysplasia: an attempt to understand the sources of variation. *Oral Oncol, 43*(3), 224-231.

La Vecchia, C. (2009). Mouthwash and oral cancer risk: an update. *Oral Oncol, 45*(3), 198-200.

La Vecchia, C., Tavani, A., Franceschi, S., Levi, F., Corrao, G., & Negri, E. (1997). Epidemiology and prevention of oral cancer. *Oral Oncol, 33*(5), 302-312.

Lam, K. Y., Ng, I. O., Yuen, A. P., Kwong, D. L., & Wei, W. (2000). Cyclin D1 expression in oral squamous cell carcinomas: clinicopathological relevance and correlation with p53 expression. *J Oral Pathol Med, 29*(4), 167-172.

Langdon, J. D., Harvey, P. W., Rapidis, A. D., Patel, M. F., Johnson, N. W., & Hopps, R. (1977). Oral cancer: the behaviour and response to treatment of 194 cases. *J Maxillofac Surg, 5*(4), 221-237.

Legate, K. R., Montanez, E., Kudlacek, O., & Fassler, R. (2006). ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat Rev Mol Cell Biol, 7*(1), 20-31.

Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature, 396*(6712), 643-649.

Li, C., Wang, J., Zhang, H., Zhu, M., Chen, F., Hu, Y., Liu, H., & Zhu, H. (2014a). Interferon-stimulated gene 15 (ISG15) is a trigger for tumorigenesis and metastasis of hepatocellular carcinoma. *Oncotarget, 5*(18), 8429-8441.

Li, Y., Rouhi, O., Chen, H., Ramirez, R., Borgia, J. A., & Deng, Y. (2014b). RNA-Seq and Network Analysis Revealed Interacting Pathways in TGF-beta-Treated Lung Cancer Cell Lines. *Cancer Inform, 13*(Suppl 5), 129-140.

Liao, C. L., Lee, M. Y., Tyan, Y. S., Kok, L. F., Wu, T. S., Koo, C. L., Wang, P. H., Chao, K. C., & Han, C. P. (2009). Progesterone receptor does not improve the performance and test effectiveness of the conventional 3-marker panel, consisting of estrogen receptor, vimentin and carcinoembryonic antigen in distinguishing between primary endocervical and endometrial adenocarcinomas in a tissue microarray extension study. *J Transl Med, 7*, 37.

Licitra, L., Grandi, C., Guzzo, M., Mariani, L., Lo Vullo, S., Valvo, F., Quattrone, P., Valagussa, P., Bonadonna, G., Molinari, R., & Cantu, G. (2003). Primary chemotherapy in resectable oral cavity squamous cell cancer: a randomized controlled trial. *J Clin Oncol, 21*(2), 327-333.

Lindenblatt Rde, C., Martinez, G. L., Silva, L. E., Faria, P. S., Camisasca, D. R., & Lourenco Sde, Q. (2012). Oral squamous cell carcinoma grading systems--analysis of the best survival predictor. *J Oral Pathol Med, 41*(1), 34-39.

Lingen, M. W., Xiao, W., Schmitt, A., Jiang, B., Pickard, R., Kreinbrink, P., Perez-Ordonez, B., Jordan, R. C., & Gillison, M. L. (2013). Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncol, 49*(1), 1-8.

Liotta, L. A. (1986). Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res, 46*(1), 1-7.

Liu, C. J., Lin, S. C., Chen, Y. J., Chang, K. M., & Chang, K. W. (2006). Array-comparative genomic hybridization to detect genomewide changes in microdissected primary and metastatic oral squamous cell carcinomas. *Mol Carcinog, 45*(10), 721-731.

Liu, G., Sengupta, P. K., Jamal, B., Yang, H. Y., Bouchie, M. P., Lindner, V., Varelas, X., & Kukuruzinska, M. A. (2013). N-glycosylation induces the CTHRC1 protein and drives oral cancer cell migration. *J Biol Chem, 288*(28), 20217-20227.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408.

Lo Muzio, L., Santarelli, A., Caltabiano, R., Rubini, C., Pieramici, T., Trevisiol, L., Carinci, F., Leonardi, R., De Lillo, A., Lanzafame, S., Bufo, P., & Piattelli, A. (2005). p63 overexpression associates with poor prognosis in head and neck squamous cell carcinoma. *Hum Pathol, 36*(2), 187-194.

Lobo, I. (2008). Chromosome abnormalities and cancer cytogenetics. *Nature Education, 1*(1), 35-43.

Lockwood, W. W., Chari, R., Chi, B., & Lam, W. L. (2006). Recent advances in array comparative genomic hybridization technologies and their applications in human genetics. *Eur J Hum Genet, 14*(2), 139-148.

Loeb, L. A. (2001). A mutator phenotype in cancer. *Cancer Res, 61*(8), 3230-3239.

Loeb, L. A., Loeb, K. R., & Anderson, J. P. (2003). Multiple mutations and cancer. *Proc Natl Acad Sci U S A, 100*(3), 776-781.

Lopez-Nieva, P., Vaquero, C., Fernandez-Navarro, P., Gonzalez-Sanchez, L., Villa-Morales, M., Santos, J., Esteller, M., & Fernandez-Piqueras, J. (2012). EPHA7, a new target gene for 6q deletion in T-cell lymphoblastic lymphomas. *Carcinogenesis, 33*(2), 452-458.

Lucito, R., Healy, J., Alexander, J., Reiner, A., Esposito, D., Chi, M., Rodgers, L., Brady, A., Sebat, J., Troge, J., West, J. A., Rostan, S., Nguyen, K. C., Powers, S., Ye, K. Q., Olshen, A., Venkatraman, E., Norton, L., & Wigler, M. (2003). Representational oligonucleotide microarray analysis: a high-resolution method to detect genome copy number variation. *Genome Res, 13*(10), 2291-2305.

Ludwig, J. A., & Weinstein, J. N. (2005). Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer, 5*(11), 845-856.

Lundahl, R. E., Foote, R. L., Bonner, J. A., Suman, V. J., Lewis, J. E., Kasperbauer, J. L., McCaffrey, T. V., & Olsen, K. D. (1998). Combined neck dissection and postoperative radiation therapy in the management of the high-risk neck: a matched-pair analysis. *Int J Radiat Oncol Biol Phys, 40*(3), 529-534.

Luukkaa, M., Vihinen, P., Kronqvist, P., Vahlberg, T., Pyrhonen, S., Kahari, V. M., & Grenman, R. (2006). Association between high collagenase-3 expression levels and poor prognosis in patients with head and neck cancer. *Head Neck, 28*(3), 225-234.

Macfarlane, G. J., Boyle, P., Evstifeeva, T. V., Robertson, C., & Scully, C. (1994). Rising trends of oral cancer mortality among males worldwide: the return of an old public health problem. *Cancer Causes Control, 5*(3), 259-265.

Macluskey, M., & Ogden, G. R. (2000). An overview of the prevention of oral cancer and diagnostic markers of malignant change: 2. Markers of value in tumour diagnosis. *Dent Update, 27*(3), 148-152.

Makinen, L. K., Hayry, V., Atula, T., Haglund, C., Keski-Santti, H., Leivo, I., Makitie, A., Passador-Santos, F., Bockelman, C., Salo, T., Sorsa, T., & Hagstrom, J. (2012). Prognostic significance of matrix metalloproteinase-2, -8, -9, and -13 in oral tongue cancer. *J Oral Pathol Med, 41*(5), 394-399.

Manning, F. C., & Patierno, S. R. (1996). Apoptosis: inhibitor or instigator of carcinogenesis? *Cancer Invest, 14*(5), 455-465.

Marquis-Nicholson, R., Aftimos, S., Hayes, I., George, A., & Love, D. R. (2010). Array comparative genomic hybridisation: a new tool in the diagnostic genetic armoury. *N Z Med J, 123*(1318), 50-61.

Marsiglia, H., Haie-Meder, C., Sasso, G., Mamelle, G., & Gerbaulet, A. (2002). Brachytherapy for T1-T2 floor-of-the-mouth cancers: the Gustave-Roussy Institute experience. *Int J Radiat Oncol Biol Phys, 52*(5), 1257-1263.

Martin, D. B., & Nelson, P. S. (2001). From genomics to proteomics: techniques and applications in cancer research. *Trends Cell Biol, 11*(11), S60-65.

Massano, J., Regateiro, F. S., Januario, G., & Ferreira, A. (2006). Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 102*(1), 67-76.

Matsubara, R., Kawano, S., Kiyosue, T., Goto, Y., Hirano, M., Jinno, T., Toyoshima, T., Kitamura, R., Oobu, K., & Nakamura, S. (2011). Increased DeltaNp63 expression is predictive of malignant transformation in oral epithelial dysplasia and poor prognosis in oral squamous cell carcinoma. *Int J Oncol, 39*(6), 1391-1399.

Matsumura, Y., Yashiro, M., Ohira, M., Tabuchi, H., & Hirakawa, K. (2005). 5-Fluorouracil up-regulates interferon pathway gene expression in esophageal cancer cells. *Anticancer Res, 25*(5), 3271-3278.

McCullough, M. J., & Farah, C. S. (2008). The role of alcohol in oral carcinogenesis with particular reference to alcohol-containing mouthwashes. *Aust Dent J, 53*(4), 302-305.

Melino, G. (2011). p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ, 18*(9), 1487-1499.

Mendelsohn, J., & Baselga, J. (2006). Epidermal growth factor receptor targeting in cancer. *Semin Oncol, 33*(4), 369-385.

Mendez, E., Houck, J. R., Doody, D. R., Fan, W., Lohavanichbutr, P., Rue, T. C., Yueh, B., Futran, N. D., Upton, M. P., Farwell, D. G., Heagerty, P. J., Zhao, L. P., Schwartz, S. M., & Chen, C. (2009). A genetic expression profile associated with oral cancer identifies a group of patients at high risk of poor survival. *Clin Cancer Res, 15*(4), 1353-1361.

Merlano, M., Benasso, M., Corvo, R., Rosso, R., Vitale, V., Blengio, F., Numico, G., Margarino, G., Bonelli, L., & Santi, L. (1996). Five-year update of a randomized trial of alternating radiotherapy and chemotherapy compared with radiotherapy alone in treatment of unresectable squamous cell carcinoma of the head and neck. *J Natl Cancer Inst, 88*(9), 583-589.

Mishra, A., & Verma, M. (2010). Cancer biomarkers: are we ready for the prime time? *Cancers (Basel), 2*(1), 190-208.

Mitelman, F., Johansson, B., Mandahl, N., & Mertens, F. (1997). Clinical significance of cytogenetic findings in solid tumors. *Cancer Genet Cytogenet, 95*(1), 1-8.

Mortazavi, H., Baharvand, M., & Mehdipour, M. (2014). Oral potentially malignant disorders: an overview of more than 20 entities. *J Dent Res Dent Clin Dent Prospects, 8*(1), 6-14.

Mufti, S. I. (1992). Alcohol acts to promote incidence of tumors. *Cancer Detect Prev, 16*(3), 157-162.

Munger, K. (2002). Disruption of oncogene/tumor suppressor networks during human carcinogenesis. *Cancer Invest, 20*(1), 71-81.

Musgrove, E. A., Caldon, C. E., Barraclough, J., Stone, A., & Sutherland, R. L. (2011). Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer, 11*(8), 558-572.

Mutation Consequences and Pathway Analysis working group of the International Cancer Genome, C. (2015). Pathway and network analysis of cancer genomes. *Nat Methods, 12*(7), 615-621.

Nagaraj, N. S. (2009). Evolving 'omics' technologies for diagnostics of head and neck cancer. *Brief Funct Genomic Proteomic, 8*(1), 49-59.

Nagpal, J. K., & Das, B. R. (2003). Oral cancer: reviewing the present understanding of its molecular mechanism and exploring the future directions for its effective management. *Oral Oncol, 39*(3), 213-221.

Nair, J., Ohshima, H., Friesen, M., Croisy, A., Bhide, S. V., & Bartsch, H. (1985). Tobacco-specific and betel nut-specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. *Carcinogenesis, 6*(2), 295-303.

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol, 11*(3), 220-228.

Neville, B. W., & Day, T. A. (2002). Oral cancer and precancerous lesions. *CA Cancer J Clin, 52*(4), 195-215.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol, 3*(3), 155-166.

Noguti, J., De Moura, C. F., De Jesus, G. P., Da Silva, V. H., Hossaka, T. A., Oshima, C. T., & Ribeiro, D. A. (2012). Metastasis from oral cancer: an overview. *Cancer Genomics Proteomics, 9*(5), 329-335.

Noutomi, Y., Oga, A., Uchida, K., Okafuji, M., Ita, M., Kawauchi, S., Furuya, T., Ueyama, Y., & Sasaki, K. (2006). Comparative genomic hybridization reveals genetic progression of oral squamous cell carcinoma from dysplasia via two different tumourigenic pathways. *J Pathol, 210*(1), 67-74.

O-Charoenrat, P., Pillai, G., Patel, S., Fisher, C., Archer, D., Eccles, S., & Rhys-Evans, P. (2003). Tumour thickness predicts cervical nodal metastases and survival in early oral tongue cancer. *Oral Oncol, 39*(4), 386-390.

Oga, A., Kong, G., Tae, K., Lee, Y., & Sasaki, K. (2001). Comparative genomic hybridization analysis reveals 3q gain resulting in genetic alteration in 3q in advanced oral squamous cell carcinoma. *Cancer Genet Cytogenet, 127*(1), 24-29.

Ogden, G. R., & Wight, A. J. (1998). Aetiology of oral cancer: alcohol. *Br J Oral Maxillofac Surg, 36*(4), 247-251.

Okey, A. B., Harper, P. A., Grant, D. M., & Hill, R. P. (1998). Chemical and radiation carcinogenesis. *The Basic Science of Oncology (Tannock, I., Hill, R., Bristow, R. and Harrington, L. eds.) New York, McGraw-Hill Inc*, 25-48.

Olayioye, M. A., Neve, R. M., Lane, H. A., & Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J, 19*(13), 3159-3167.

Oliveira, L. R., Ribeiro-Silva, A., Costa, J. P., Simoes, A. L., Matteo, M. A., & Zucoloto, S. (2008). Prognostic factors and survival analysis in a sample of oral squamous cell carcinoma patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 106*(5), 685-695.

Oliver, C. L., Bauer, J. A., Wolter, K. G., Ubell, M. L., Narayan, A., O'Connell, K. M., Fisher, S. G., Wang, S., Wu, X., Ji, M., Carey, T. E., & Bradford, C. R. (2004). In vitro effects of the BH3 mimetic, (-)-gossypol, on head and neck squamous cell carcinoma cells. *Clin Cancer Res, 10*(22), 7757-7763.

Omar, E. A. (2013). The Outline of Prognosis and New Advances in Diagnosis of Oral Squamous Cell Carcinoma (OSCC): Review of the Literature. *Journal of Oral Oncology, 2013*, 13.

Omar, Z. A., & Tamin, N. S. I. (2011). National Cancer Registry Report 2007. *Malaysia Cancer Statistics-Data and Figure, Kuala Lumpur: National Cancer Registry, Ministry of Health Malaysia*, 89.

Oren, M. (1992). The involvement of oncogenes and tumor suppressor genes in the control of apoptosis. *Cancer Metastasis Rev, 11*(2), 141-148.

P, O. C., Rhys-Evans, P. H., & Eccles, S. A. (2001). Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg, 127*(7), 813-820.

Pacifico, A., & Leone, G. (2007). Role of p53 and CDKN2A inactivation in human squamous cell carcinomas. *J Biomed Biotechnol, 2007*(3), 43418.

Parisi, E., & Glick, M. (2005). Cervical lymphadenopathy in the dental patient: a review of clinical approach. *Quintessence Int, 36*(6), 423-436.

Park, J. Y., Muscat, J. E., Ren, Q., Schantz, S. P., Harwick, R. D., Stern, J. C., Pike, V., Richie, J. P., Jr., & Lazarus, P. (1997). CYP1A1 and GSTM1 polymorphisms and oral cancer risk. *Cancer Epidemiol Biomarkers Prev, 6*(10), 791-797.

Patel, A. S., Schechter, G. L., Wasilenko, W. J., & Somers, K. D. (1998). Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion in vitro. *Oncogene, 16*(25), 3227-3232.

Patel, S. G., & Shah, J. P. (2005). TNM staging of cancers of the head and neck: striving for uniformity among diversity. *CA Cancer J Clin, 55*(4), 242-258; quiz 261-242, 264.

Peng, C. H., Liao, C. T., Peng, S. C., Chen, Y. J., Cheng, A. J., Juang, J. L., Tsai, C. Y., Chen, T. C., Chuang, Y. J., Tang, C. Y., Hsieh, W. P., & Yen, T. C. (2011). A novel molecular signature identified by systems genetics approach predicts prognosis in oral squamous cell carcinoma. *PLoS One, 6*(8), e23452.

Petridou, E., Zavras, A. I., Lefatzis, D., Dessypris, N., Laskaris, G., Dokianakis, G., Segas, J., Douglas, C. W., Diehl, S. R., & Trichopoulos, D. (2002). The role of diet and specific micronutrients in the etiology of oral carcinoma. *Cancer, 94*(11), 2981-2988.

Petti, S. (2003). Pooled estimate of world leukoplakia prevalence: a systematic review. *Oral Oncol, 39*(8), 770-780.

Petti, S. (2009). Lifestyle risk factors for oral cancer. *Oral Oncol, 45*(4-5), 340-350.

Pezzella, F., Turley, H., Kuzu, I., Tungekar, M. F., Dunnill, M. S., Pierce, C. B., Harris, A., Gatter, K. C., & Mason, D. Y. (1993). bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med, 329*(10), 690-694.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu Rev Biochem, 70*, 503-533.

Pickering, C. R., Zhang, J., Yoo, S. Y., Bengtsson, L., Moorthy, S., Neskey, D. M., Zhao, M., Ortega Alves, M. V., Chang, K., Drummond, J., Cortez, E., Xie, T. X., Zhang, D., Chung, W., Issa, J. P., Zweidler-McKay, P. A., Wu, X., El-Naggar, A. K., Weinstein, J. N., Wang, J., Muzny, D. M., Gibbs, R. A., Wheeler, D. A., Myers, J. N., & Frederick, M. J. (2013). Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. *Cancer Discov, 3*(7), 770-781.

Pindborg, J. J., Reichart, P.A., Smith, C.J., van der Waal, I. (1997). Histological Typing of Cancer and Precancer of the Oral Mucosa. New York: Springer-Verlag Berlin Heidelberg.

Pinkel, D., & Albertson, D. G. (2005). Array comparative genomic hybridization and its applications in cancer. *Nat Genet, 37 Suppl*, S11-17.

Pon, J. R., & Marra, M. A. (2015). Driver and passenger mutations in cancer. *Annu Rev Pathol, 10*, 25-50.

Przybytkowski, E., Ferrario, C., & Basik, M. (2011). The use of ultra-dense array CGH analysis for the discovery of micro-copy number alterations and gene fusions in the cancer genome. *BMC Med Genomics, 4*, 16.

Que, L., Zhao, D., Tang, X. F., Liu, J. Y., Zhang, X. Y., Zhan, Y. H., & Zhang, L. (2016). Effects of lentivirus-mediated shRNA targeting integrin-linked kinase on oral squamous cell carcinoma in vitro and in vivo. *Oncol Rep, 35*(1), 89-98.

Redon, R., Hussenet, T., Bour, G., Caulee, K., Jost, B., Muller, D., Abecassis, J., & du Manoir, S. (2002). Amplicon mapping and transcriptional analysis pinpoint cyclin L as a candidate oncogene in head and neck cancer. *Cancer Res, 62*(21), 6211-6217.

Reed, J. A., Lin, Q., Chen, D., Mian, I. S., & Medrano, E. E. (2005). SKI pathways inducing progression of human melanoma. *Cancer Metastasis Rev, 24*(2), 265-272.

Reshmi, S. C., & Gollin, S. M. (2005). Chromosomal instability in oral cancer cells. *J Dent Res, 84*(2), 107-117.

Resnier, P., Montier, T., Mathieu, V., Benoit, J. P., & Passirani, C. (2013). A review of the current status of siRNA nanomedicines in the treatment of cancer. *Biomaterials, 34*(27), 6429-6443.

Reuschenbach, M., Kansy, K., Garbe, K., Vinokurova, S., Flechtenmacher, C., Toth, C., Prigge, E. S., Thiele, O. C., Reinert, S., Hoffmann, J., von Knebel Doeberitz, M., & Freier, K. (2013). Lack of evidence of human papillomavirus-induced squamous cell carcinomas of the oral cavity in southern Germany. *Oral Oncol, 49*(9), 937-942.

Reuter, C. W., Morgan, M. A., & Eckardt, A. (2007). Targeting EGF-receptor-signalling in squamous cell carcinomas of the head and neck. *Br J Cancer, 96*(3), 408-416.

Ribeiro, D. C., Gleber-Netto, F. O., Sousa, S. F., Bernardes, V. D., Guimaraes-Abreu, M. H., & Aguiar, M. C. (2012). Immunohistochemical expression of EGFR in oral leukoplakia: association with clinicopathological features and cellular proliferation. *Med Oral Patol Oral Cir Bucal, 17*(5), e739-744.

Rodrigo, J. P., Garcia, L. A., Ramos, S., Lazo, P. S., & Suarez, C. (2000). EMS1 gene amplification correlates with poor prognosis in squamous cell carcinomas of the head and neck. *Clin Cancer Res, 6*(8), 3177-3182.

Rodriguez, T., Altieri, A., Chatenoud, L., Gallus, S., Bosetti, C., Negri, E., Franceschi, S., Levi, F., Talamini, R., & La Vecchia, C. (2004). Risk factors for oral and pharyngeal cancer in young adults. *Oral Oncol, 40*(2), 207-213.

Rodu, B., & Jansson, C. (2004). Smokeless tobacco and oral cancer: a review of the risks and determinants. *Crit Rev Oral Biol Med, 15*(5), 252-263.

Rogers, S. J., Harrington, K. J., Rhys-Evans, P., P, O. C., & Eccles, S. A. (2005). Biological significance of c-erbB family oncogenes in head and neck cancer. *Cancer Metastasis Rev, 24*(1), 47-69.

Roh, H. J., Shin, D. M., Lee, J. S., Ro, J. Y., Tainsky, M. A., Hong, W. K., & Hittelman, W. N. (2000). Visualization of the timing of gene amplification during multistep head and neck tumorigenesis. *Cancer Res, 60*(22), 6496-6502.

Rowland, I. R. (1991). *Nutrition, toxicity, and cancer*. Boca Raton, Florida: CRC Press.

Rowley, H., Jones, A., Spandidos, D., & Field, J. (1996). Definition of a tumor suppressor gene locus on the short arm of chromosome 3 in squamous cell carcinoma of the head and neck by means of microsatellite markers. *Arch Otolaryngol Head Neck Surg, 122*(5), 497-501.

Roz, L., Wu, C. L., Porter, S., Scully, C., Speight, P., Read, A., Sloan, P., & Thakker, N. (1996). Allelic imbalance on chromosome 3p in oral dysplastic lesions: an early event in oral carcinogenesis. *Cancer Res, 56*(6), 1228-1231.

Ruiz, C., Martins, J. R., Rudin, F., Schneider, S., Dietsche, T., Fischer, C. A., Tornillo, L., Terracciano, L. M., Schreiber, R., Bubendorf, L., & Kunzelmann, K. (2012). Enhanced expression of ANO1 in head and neck squamous cell carcinoma causes cell migration and correlates with poor prognosis. *PLoS One, 7*(8), e43265.

Russo, A., Migliavacca, M., Zanna, I., Macaluso, M., Gebbia, N., & Bazan, V. (2006). Caretakers and Gatekeepers. *In: Encyclopedia of Life Sciences, John Wiley & Sons*.

Sager, R. (1989). Tumor suppressor genes: the puzzle and the promise. *Science, 246*(4936), 1406-1412.

Salahshourifar, I., Vincent-Chong, V. K., Kallarakkal, T. G., & Zain, R. B. (2014). Genomic DNA copy number alterations from precursor oral lesions to oral squamous cell carcinoma. *Oral Oncol, 50*(5), 404-412.

Sankaranarayanan, R., Ramadas, K., Amarasinghe, H., Subramanian, S., & Johnson, N. (2015). Cancer: Disease Control Priorities. In H. Gelband, Jha, P., Sankaranarayanan (Ed.), *Oral Cancer: Prevention, Early Detection, and Treatment* (Vol. 3). Washington (DC): The International Bank for Reconstruction and Development / The World Bank; .

Sankaranarayanan, R., Swaminathan, R., Jayant, K., & Brenner, H. (2011). An overview of cancer survival in Africa, Asia, the Caribbean and Central America: the case for investment in cancer health services. *IARC Sci Publ*(162), 257-291.

Sant, M., Allemani, C., Santaquilani, M., Knijn, A., Marchesi, F., Capocaccia, R., & Group, E. W. (2009). EUROCARE-4. Survival of cancer patients diagnosed in 1995-1999. Results and commentary. *Eur J Cancer, 45*(6), 931-991.

Sato, M., Sato, T., Izumo, T., & Amagasa, T. (1999). Genetic polymorphism of drug-metabolizing enzymes and susceptibility to oral cancer. *Carcinogenesis, 20*(10), 1927-1931.

Saunders, W. S., Shuster, M., Huang, X., Gharaibeh, B., Enyenihi, A. H., Petersen, I., & Gollin, S. M. (2000). Chromosomal instability and cytoskeletal defects in oral cancer cells. *Proc Natl Acad Sci U S A, 97*(1), 303-308.

Sawair, F. A., Irwin, C. R., Gordon, D. J., Leonard, A. G., Stephenson, M., & Napier, S. S. (2003). Invasive front grading: reliability and usefulness in the management of oral squamous cell carcinoma. *J Oral Pathol Med, 32*(1), 1-9.

Sawazaki-Calone, I., Rangel, A., Bueno, A. G., Morais, C. F., Nagai, H. M., Kunz, R. P., Souza, R. L., Rutkauskis, L., Salo, T., Almangush, A., & Coletta, R. D. (2015). The prognostic value of histopathological grading systems in oral squamous cell carcinomas. *Oral Dis, 21*(6), 755-761.

Schvartzman, J. M., Sotillo, R., & Benezra, R. (2010). Mitotic chromosomal instability and cancer: mouse modelling of the human disease. *Nat Rev Cancer, 10*(2), 102-115.

Scully, C., & Bagan, J. (2009). Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. *Oral Dis, 15*(6), 388-399.

Scully, C., Field, J. K., & Tanzawa, H. (2000). Genetic aberrations in oral or head and neck squamous cell carcinoma 2: chromosomal aberrations. *Oral Oncol, 36*(4), 311-327.

Senchenko, V. N., Krasnov, G. S., Dmitriev, A. A., Kudryavtseva, A. V., Anedchenko, E. A., Braga, E. A., Pronina, I. V., Kondratieva, T. T., Ivanov, S. V., Zabarovsky, E. R., & Lerman, M. I. (2011). Differential expression of CHL1 gene during development of major human cancers. *PLoS One, 6*(3), e15612.

Serrano, M., Hannon, G. J., & Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature, 366*(6456), 704-707.

Shah, J. P. (1990). Cervical lymph node metastases--diagnostic, therapeutic, and prognostic implications. *Oncology (Williston Park), 4*(10), 61-69; discussion 72, 76.

Shahnavaz, S. A., Regezi, J. A., Bradley, G., Dube, I. D., & Jordan, R. C. (2000). p53 gene mutations in sequential oral epithelial dysplasias and squamous cell carcinomas. *J Pathol, 190*(4), 417-422.

Shay, J. W., & Wright, W. E. (2006). Telomerase therapeutics for cancer: challenges and new directions. *Nat Rev Drug Discov, 5*(7), 577-584.

Sheu, J. J., Hua, C. H., Wan, L., Lin, Y. J., Lai, M. T., Tseng, H. C., Jinawath, N., Tsai, M. H., Chang, N. W., Lin, C. F., Lin, C. C., Hsieh, L. J., Wang, T. L., Shih Ie, M., & Tsai, F. J. (2009). Functional genomic analysis identified epidermal growth factor receptor activation as the most common genetic event in oral squamous cell carcinoma. *Cancer Res, 69*(6), 2568-2576.

Shiboski, C. H., Schmidt, B. L., & Jordan, R. C. (2005). Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. *Cancer, 103*(9), 1843-1849.

Shinawi, M., & Cheung, S. W. (2008). The array CGH and its clinical applications. *Drug Discov Today, 13*(17-18), 760-770.

Siebers, T. J., Merkx, M. A., Slootweg, P. J., Melchers, W. J., van Cleef, P., & de Wilde, P. C. (2008). No high-risk HPV detected in SCC of the oral tongue in the absolute absence of tobacco and alcohol--a case study of seven patients. *Oral Maxillofac Surg, 12*(4), 185-188.

Silverman, S., Jr. (2001). Demographics and occurrence of oral and pharyngeal cancers. The outcomes, the trends, the challenge. *J Am Dent Assoc, 132 Suppl*, 7S-11S.

Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S. D., Willis, J., Dawson, D., Willson, J. K., Gazdar, A. F., Hartigan, J., Wu, L., Liu, C., Parmigiani, G., Park, B. H., Bachman, K. E., Papadopoulos, N., Vogelstein, B., Kinzler, K. W., & Velculescu, V. E. (2006). The consensus coding sequences of human breast and colorectal cancers. *Science, 314*(5797), 268-274.

Snijders, A. M., Schmidt, B. L., Fridlyand, J., Dekker, N., Pinkel, D., Jordan, R. C., & Albertson, D. G. (2005). Rare amplicons implicate frequent deregulation of cell fate specification pathways in oral squamous cell carcinoma. *Oncogene, 24*(26), 4232-4242.

Sobin, L. H., Gospodarowicz, M. K., & Wittekind, C. (2011). *TNM classification of malignant tumours* (7th ed.). Hoboken, New Jersey: John Wiley & Sons.

Song, G., Ouyang, G., & Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med, 9*(1), 59-71.

Song, L., Liu, L., Wu, Z., Lin, C., Dai, T., Yu, C., Wang, X., Wu, J., Li, M., & Li, J. (2012). Knockdown of stomatin-like protein 2 (STOML2) reduces the invasive ability of glioma cells through inhibition of the NF-kappaB/MMP-9 pathway. *J Pathol, 226*(3), 534-543.

Speight, P. M. (2007). Update on oral epithelial dysplasia and progression to cancer. *Head Neck Pathol, 1*(1), 61-66.

Sporn, M. B. (1996). The war on cancer. *Lancet, 347*(9012), 1377-1381.

Sreelekha, T. T., Ramadas, K., Pandey, M., Thomas, G., Nalinakumari, K. R., & Pillai, M. R. (2001). Genetic polymorphism of CYP1A1, GSTM1 and GSTT1 genes in Indian oral cancer. *Oral Oncol, 37*(7), 593-598.

Sticht, C., Hofele, C., Flechtenmacher, C., Bosch, F. X., Freier, K., Lichter, P., & Joos, S. (2005). Amplification of Cyclin L1 is associated with lymph node metastases in head and neck squamous cell carcinoma (HNSCC). *Br J Cancer, 92*(4), 770-774.

Stransky, N., Egloff, A. M., Tward, A. D., Kostic, A. D., Cibulskis, K., Sivachenko, A., Kryukov, G. V., Lawrence, M. S., Sougnez, C., McKenna, A., Shefler, E., Ramos, A. H., Stojanov, P., Carter, S. L., Voet, D., Cortes, M. L., Auclair, D., Berger, M. F., Saksena, G., Guiducci, C., Onofrio, R. C., Parkin, M., Romkes, M., Weissfeld, J. L., Seethala, R. R., Wang, L., Rangel-Escareno, C., Fernandez-Lopez, J. C., Hidalgo-Miranda, A., Melendez-Zajgla, J., Winckler, W., Ardlie, K., Gabriel, S. B., Meyerson, M., Lander, E. S., Getz, G., Golub, T. R., Garraway, L. A., & Grandis, J. R. (2011). The mutational landscape of head and neck squamous cell carcinoma. *Science, 333*(6046), 1157-1160.

Stratton, M. R., Campbell, P. J., & Futreal, P. A. (2009). The cancer genome. *Nature, 458*(7239), 719-724.

Su, X., Chakravarti, D., & Flores, E. R. (2013). p63 steps into the limelight: crucial roles in the suppression of tumorigenesis and metastasis. *Nat Rev Cancer, 13*(2), 136-143.

Sudbo, J., Bankfalvi, A., Bryne, M., Marcelpoil, R., Boysen, M., Piffko, J., Hemmer, J., Kraft, K., & Reith, A. (2000). Prognostic value of graph theory-based tissue architecture analysis in carcinomas of the tongue. *Lab Invest, 80*(12), 1881-1889.

Sumino, J., Uzawa, N., Okada, N., Miyaguchi, K., Mogushi, K., Takahashi, K., Sato, H., Michikawa, C., Nakata, Y., Tanaka, H., & Amagasa, T. (2013). Gene expression changes in initiation and progression of oral squamous cell carcinomas revealed by laser microdissection and oligonucleotide microarray analysis. *Int J Cancer, 132*(3), 540-548.

Swaminathan, R., Lucas, E., & Sankaranarayanan, R. (2011). Cancer survival in Africa, Asia, the Caribbean and Central America: database and attributes. *IARC Sci Publ*(162), 23-31.

Tameda, M., Sugimoto, K., Shiraki, K., Yamamoto, N., Okamoto, R., Usui, M., Ito, M., Takei, Y., Nobori, T., Kojima, T., Suzuki, H., Uchida, M., & Uchida, K. (2014). Collagen triple helix repeat containing 1 is overexpressed in hepatocellular carcinoma and promotes cell proliferation and motility. *Int J Oncol, 45*(2), 541-548.

Tang, L., Dai, D. L., Su, M., Martinka, M., Li, G., & Zhou, Y. (2006). Aberrant expression of collagen triple helix repeat containing 1 in human solid cancers. *Clin Cancer Res, 12*(12), 3716-3722.

Tang, M. R., Wang, Y. X., Guo, S., Han, S. Y., & Wang, D. (2012). CSMD1 exhibits antitumor activity in A375 melanoma cells through activation of the Smad pathway. *Apoptosis, 17*(9), 927-937.

Tankere, F., Camproux, A., Barry, B., Guedon, C., Depondt, J., & Gehanno, P. (2000). Prognostic value of lymph node involvement in oral cancers: a study of 137 cases. *Laryngoscope, 110*(12), 2061-2065.

Taoudi Benchekroun, M., Saintigny, P., Thomas, S. M., El-Naggar, A. K., Papadimitrakopoulou, V., Ren, H., Lang, W., Fan, Y. H., Huang, J., Feng, L., Lee, J. J., Kim, E. S., Hong, W. K., Johnson, F. M., Grandis, J. R., & Mao, L. (2010). Epidermal growth factor receptor expression and gene copy number in the risk of oral cancer. *Cancer Prev Res (Phila), 3*(7), 800-809.

TCGA. (2015). Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature, 517*(7536), 576-582.

Thangaraj, S. V., Shyamsundar, V., Krishnamurthy, A., Ramani, P., Ganesan, K., Muthuswami, M., & Ramshankar, V. (2016). Molecular Portrait of Oral Tongue Squamous Cell Carcinoma Shown by Integrative Meta-Analysis of Expression Profiles with Validations. *PLoS One, 11*(6), e0156582.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer, 2*(6), 442-454.

Todd, R., Chou, M. Y., Matossian, K., Gallagher, G. T., Donoff, R. B., & Wong, D. T. (1991). Cellular sources of transforming growth factor-alpha in human oral cancer. *J Dent Res, 70*(5), 917-923.

Todd, R., Donoff, R. B., & Wong, D. T. (1997). The molecular biology of oral carcinogenesis: toward a tumor progression model. *J Oral Maxillofac Surg, 55*(6), 613-623; discussion 623-615.

Towle, R., Tsui, I. F., Zhu, Y., MacLellan, S., Poh, C. F., & Garnis, C. (2014). Recurring DNA copy number gain at chromosome 9p13 plays a role in the activation of multiple candidate oncogenes in progressing oral premalignant lesions. *Cancer Med, 3*(5), 1170-1184.

Tsui, I. F., Poh, C. F., Garnis, C., Rosin, M. P., Zhang, L., & Lam, W. L. (2009). Multiple pathways in the FGF signaling network are frequently deregulated by gene amplification in oral dysplasias. *Int J Cancer, 125*(9), 2219-2228.

Uchida, K., Oga, A., Nakao, M., Mano, T., Mihara, M., Kawauchi, S., Furuya, T., Ueyama, Y., & Sasaki, K. (2011). Loss of 3p26.3 is an independent prognostic factor in patients with oral squamous cell carcinoma. *Oncol Rep, 26*(2), 463-469.

Udagawa, T., Fernandez, A., Achilles, E. G., Folkman, J., & D'Amato, R. J. (2002). Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of tumor dormancy. *FASEB J, 16*(11), 1361-1370.

van der Waal, I. (2009). Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncol, 45*(4-5), 317-323.

van Hooff, S. R., Leusink, F. K., Roepman, P., Baatenburg de Jong, R. J., Speel, E. J., van den Brekel, M. W., van Velthuysen, M. L., van Diest, P. J., van Es, R. J., Merkx, M. A., Kummer, J. A., Leemans, C. R., Schuuring, E., Langendijk, J. A., Lacko, M., De Herdt, M. J., Jansen, J. C., Brakenhoff, R. H., Slootweg, P. J., Takes, R. P., & Holstege, F. C. (2012). Validation of a gene expression signature for assessment of lymph node metastasis in oral squamous cell carcinoma. *J Clin Oncol, 30*(33), 4104-4110.

Venkatraman, E. S., & Olshen, A. B. (2007). A faster circular binary segmentation algorithm for the analysis of array CGH data. *Bioinformatics, 23*(6), 657-663.

Viet, C. T., & Schmidt, B. L. (2010). Understanding oral cancer in the genome era. *Head Neck, 32*(9), 1246-1268.

Vijayakumar, M., Burrah, R., Sabitha, K. S., Nadimul, H., & Rajani, B. C. (2011). To operate or not to operate n0 neck in early cancer of the tongue? A prospective study. *Indian J Surg Oncol, 2*(3), 172-175.

Vincent-Chong, V. K., Anwar, A., Karen-Ng, L. P., Cheong, S. C., Yang, Y. H., Pradeep, P. J., Rahman, Z. A., Ismail, S. M., Zaini, Z. M., Prepageran, N., Kallarakkal, T. G., Ramanathan, A., Mohayadi, N. A., Rosli, N. S., Mustafa, W. M., Abraham, M. T., Tay, K. K., & Zain, R. B. (2013). Genome wide analysis of chromosomal alterations in oral squamous cell carcinomas revealed over expression of MGAM and ADAM9. *PLoS One, 8*(2), e54705.

Vincent-Chong, V. K., Ismail, S. M., Rahman, Z. A., Sharifah, N. A., Anwar, A., Pradeep, P. J., Ramanathan, A., Karen-Ng, L. P., Kallarakkal, T. G., Mustafa, W. M., Abraham, M. T., Tay, K. K., & Zain, R. B. (2012). Genome-wide analysis of oral squamous cell carcinomas revealed over expression of ISG15, Nestin and WNT11. *Oral Dis, 18*(5), 469-476.

Vincent-Chong, V. K., Salahshourifar, I., Razali, R., Anwar, A., & Zain, R. B. (2016). Immortalization of epithelial cells in oral carcinogenesis as revealed by genome-wide array comparative genomic hybridization: A meta-analysis. *Head Neck, 38* (Suppl 1), E783-797.

Virgilio, L., Shuster, M., Gollin, S. M., Veronese, M. L., Ohta, M., Huebner, K., & Croce, C. M. (1996). FHIT gene alterations in head and neck squamous cell carcinomas. *Proc Natl Acad Sci U S A, 93*(18), 9770-9775.

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A., Jr., & Kinzler, K. W. (2013). Cancer genome landscapes. *Science, 339*(6127), 1546-1558.

Warnakulasuriya, S. (2009a). Causes of oral cancer--an appraisal of controversies. *Br Dent J, 207*(10), 471-475.

Warnakulasuriya, S. (2009b). Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol, 45*(4-5), 309-316.

Warnakulasuriya, S. (2014a). Prognostic and predictive markers for oral squamous cell carcinoma: The importance of clinical, pathological and molecular markers. *Saudi Journal of Medicine and Medical Sciences, 2*(1), 12.

Warnakulasuriya, S. (2014b). Prognostic and predictive markers for oral squamous cell carcinoma: The importance of clinical, pathological and molecular markers. *Saudi Journal of Medicine and Medical Sciences, 2*(1), 12-16.

Warnakulasuriya, S., Johnson, N. W., & van der Waal, I. (2007). Nomenclature and classification of potentially malignant disorders of the oral mucosa. *J Oral Pathol Med, 36*(10), 575-580.

Warnakulasuriya, S., Reibel, J., Bouquot, J., & Dabelsteen, E. (2008). Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. *J Oral Pathol Med, 37*(3), 127-133.

Warnakulasuriya, S., Sutherland, G., & Scully, C. (2005). Tobacco, oral cancer, and treatment of dependence. *Oral Oncol, 41*(3), 244-260.

Weinberg, R. A. (1991). Tumor suppressor genes. *Science, 254*(5035), 1138-1146.

Weinberg, R. A. (1996). How cancer arises. *Sci Am, 275*(3), 62-70.

Weinstein, I. B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. *Science, 297*(5578), 63-64.

Wilczynski, S. P., Lin, B. T., Xie, Y., & Paz, I. B. (1998). Detection of human papillomavirus DNA and oncoprotein overexpression are associated with distinct morphological patterns of tonsillar squamous cell carcinoma. *Am J Pathol, 152*(1), 145-156.

Wilkey, J. F., Buchberger, G., Saucier, K., Patel, S. M., Eisenberg, E., Nakagawa, H., Michaylira, C. Z., Rustgi, A. K., & Mallya, S. M. (2009). Cyclin D1 overexpression increases susceptibility to 4-nitroquinoline-1-oxide-induced dysplasia and neoplasia in murine squamous oral epithelium. *Mol Carcinog, 48*(9), 853-861.

Williams, M. D. (2010). Integration of biomarkers including molecular targeted therapies in head and neck cancer. *Head Neck Pathol, 4*(1), 62-69.

Winn, D. M., Blot, W. J., McLaughlin, J. K., Austin, D. F., Greenberg, R. S., Preston-Martin, S., Schoenberg, J. B., & Fraumeni, J. F., Jr. (1991). Mouthwash use and oral conditions in the risk of oral and pharyngeal cancer. *Cancer Res, 51*(11), 3044-3047.

Woolgar, J. A. (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol, 42*(3), 229-239.

Woolgar, J. A., Rogers, S., West, C. R., Errington, R. D., Brown, J. S., & Vaughan, E. D. (1999). Survival and patterns of recurrence in 200 oral cancer patients treated by radical surgery and neck dissection. *Oral Oncol, 35*(3), 257-265.

Woolgar, J. A., & Scott, J. (1995). Prediction of cervical lymph node metastasis in squamous cell carcinoma of the tongue/floor of mouth. *Head Neck, 17*(6), 463-472.

Wreesmann, V. B., Shi, W., Thaler, H. T., Poluri, A., Kraus, D. H., Pfister, D., Shaha, A. R., Shah, J. P., Rao, P. H., & Singh, B. (2004). Identification of novel prognosticators of outcome in squamous cell carcinoma of the head and neck. *J Clin Oncol, 22*(19), 3965-3972.

Xi, S., & Grandis, J. R. (2003). Gene therapy for the treatment of oral squamous cell carcinoma. *J Dent Res, 82*(1), 11-16.

Xia, W., Lau, Y. K., Zhang, H. Z., Liu, A. R., Li, L., Kiyokawa, N., Clayman, G. L., Katz, R. L., & Hung, M. C. (1997). Strong correlation between c-erbB-2 overexpression and overall survival of patients with oral squamous cell carcinoma. *Clin Cancer Res, 3*(1), 3-9.

Xu, C., Liu, Y., Wang, P., Fan, W., Rue, T. C., Upton, M. P., Houck, J. R., Lohavanichbutr, P., Doody, D. R., Futran, N. D., Zhao, L. P., Schwartz, S. M., Chen, C., & Mendez, E. (2010). Integrative analysis of DNA copy number and gene expression in metastatic oral squamous cell carcinoma identifies genes associated with poor survival. *Mol Cancer, 9*, 143.

Yamamoto, S., Tomita, Y., Hoshida, Y., Iizuka, N., Kidogami, S., Miyata, H., Takiguchi, S., Fujiwara, Y., Yasuda, T., Yano, M., Nakamori, S., Sakon, M., Monden, M., & Aozasa, K. (2004). Expression level of valosin-containing protein (p97) is associated with prognosis of esophageal carcinoma. *Clin Cancer Res, 10*(16), 5558-5565.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., & McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature, 398*(6729), 714-718.

Yanik, E. L., Katki, H. A., Silverberg, M. J., Manos, M. M., Engels, E. A., & Chaturvedi, A. K. (2015). Leukoplakia, Oral Cavity Cancer Risk, and Cancer Survival in the U.S. Elderly. *Cancer Prev Res (Phila), 8*(9), 857-863.

Yano, S., Kondo, K., Yamaguchi, M., Richmond, G., Hutchison, M., Wakeling, A., Averbuch, S., & Wadsworth, P. (2003). Distribution and function of EGFR in human tissue and the effect of EGFR tyrosine kinase inhibition. *Anticancer Res, 23*(5A), 3639-3650.

Yokota, J., & Sugimura, T. (1993). Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. *FASEB J, 7*(10), 920-925.

Yuen, A. P., Lam, K. Y., Wei, W. I., Lam, K. Y., Ho, C. M., Chow, T. L., & Yuen, W. F. (2000). A comparison of the prognostic significance of tumor diameter, length, width, thickness, area, volume, and clinicopathological features of oral tongue carcinoma. *Am J Surg, 180*(2), 139-143.

Zain, R. B., Athirajan, V., Ghani, W. M., Razak, I. A., Raja Latifah, R. J., Ismail, S. M., Sallam, A. A., Bustam, A. Z., Rahman, Z. A., Hussien, A., Talib, N., Cheong, S. C., & Jallaludin, A. (2013). An oral cancer biobank initiative: a platform for multidisciplinary research in a developing country. *Cell Tissue Bank, 14*(1), 45-52.

Zain, R. B., Ikeda, N., Razak, I. A., Axell, T., Majid, Z. A., Gupta, P. C., & Yaacob, M. (1997). A national epidemiological survey of oral mucosal lesions in Malaysia. *Community Dent Oral Epidemiol, 25*(5), 377-383.

Zelefsky, M. J., Harrison, L. B., Fass, D. E., Armstrong, J., Spiro, R. H., Shah, J. P., & Strong, E. W. (1990). Postoperative radiotherapy for oral cavity cancers: impact of anatomic subsite on treatment outcome. *Head Neck, 12*(6), 470-475.

Zhang, D., & Zhang, D. E. (2011). Interferon-stimulated gene 15 and the protein ISGylation system. *J Interferon Cytokine Res, 31*(1), 119-130.

Zhang, L., Poh, C. F., Williams, M., Laronde, D. M., Berean, K., Gardner, P. J., Jiang, H., Wu, L., Lee, J. J., & Rosin, M. P. (2012). Loss of heterozygosity (LOH) profiles--validated risk predictors for progression to oral cancer. *Cancer Prev Res (Phila), 5*(9), 1081-1089.

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res, 12*(1), 9-18.

Zhao, Y., Yu, D., Li, H., Nie, P., Zhu, Y., Liu, S., Zhu, M., & Fang, B. (2014). Cyclin D1 overexpression is associated with poor clinicopathological outcome and survival in oral squamous cell carcinoma in Asian populations: insights from a meta-analysis. *PLoS One, 9*(3), e93210.

Zhu, Q., Krakowski, A. R., Dunham, E. E., Wang, L., Bandyopadhyay, A., Berdeaux, R., Martin, G. S., Sun, L., & Luo, K. (2007). Dual role of SnoN in mammalian tumorigenesis. *Mol Cell Biol, 27*(1), 324-339.

Zimmermann, M., Zouhair, A., Azria, D., & Ozsahin, M. (2006). The epidermal growth factor receptor (EGFR) in head and neck cancer: its role and treatment implications. *Radiat Oncol, 1*, 11.

Zlobec, I., Steele, R., Terracciano, L., Jass, J. R., & Lugli, A. (2007). Selecting immunohistochemical cut-off scores for novel biomarkers of progression and survival in colorectal cancer. *J Clin Pathol, 60*(10), 1112-1116.

zur Hausen, H. (1996). Papillomavirus infections-a major cause of human cancers. *Biochim Biophys Acta, 1288*(2), F55-78.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications

1. Vincent-Chong, V. K., Anwar, A., Karen-Ng, L. P., Cheong, S. C., Yang, Y. H., Pradeep, P. J., et al. (2013). Genome-wide analysis of chromosomal alterations in oral squamous cell carcinomas revealed over-expression of MGAM and ADAM9. PLoS One, 8(2), e54705.

2. Vincent-Chong, V. K., Salahshourifar, I., Karen-Ng, L. P., Siow, M. Y., Kallarakkal, T. G., Ramanathan, A., et al. (2014). Over-expression of MMP13 is associated with clinical outcomes and poor prognosis in oral squamous cell carcinoma. ScientificWorldJournal, 2014, 897523.

3. Vincent-Chong, V. K., Salahshourifar, I., Razali, R., Anwar, A., & Zain, R. B. (2015). Immortalization of epithelial cells in oral carcinogenesis as revealed by genome-wide array comparative genomic hybridization: A meta-analysis. Head Neck.

4. Salahshourifar, I., Vincent-Chong, V. K., Kallarakkal, T. G., & Zain, R. B. (2014). Genomic DNA copy number alterations from precursor oral lesions to oral squamous cell carcinoma. Oral Oncol, 50(5), 404-412.

List of paper presented

1. Vincent-Chong VK. Meta-analysis of genome-wide profiling in oral squamous cell carcinoma. Presented: 28th Annual Scientific Meeting of the International Association for Dental Research Southeast Asian Division, from August 13 to August 14, 2014 Kuching, Malaysia.
2. Vincent-Chong VK. Reduced expression of ISG15 by small interfering RNA inhibits cell proliferation of gingival squamous cell carcinoma. Presented: 5th International Academy of Oral Oncology, from July 8 to 11 July, 2015 at Sao Paolo, Brazil.
3. Vincent-Chong VK. Genome-wide analysis of chromosomal alterations identified interferon stimulated gene 15 (ISG15) as a driver gene for tumourigenesis and metastasis of oral squamous cell carcinomas. Presented: International Postgraduate Research Awards Seminar from March 7 to March 8, 2016 at University of Malaya, Kuala Lumpur, Malaysia.

APPENDIX

APPENDIX A: DETAIL OF DNEASY BLOOD AND TISSUE KIT EXTRACTION (QIAGEN GMBH GERMANY) PROTOCOL

All tumour tissues for test gDNA samples were isolated by using DNEasy Blood & Tissue Kit (Qiagen GmBH Germany) following the manufacturer's instructions. 750 µm of the tissue sections were mixed with 1x PBS buffer and then centrifuged in 5000rpm for 5 minutes. The supernatant were discarded thereafter. Then, 180µl of ATL buffer and 20 µl of proteinase K were added and then kept for incubation at 56º C, overnight. Later, 200 µl AL buffer was added again and then followed by incubation at 70° C for 10 minutes. Afterthat, absolute ethanol (200 µl) were added and thereafter all the mixtures were transferred to QIAamp Mini spin for centrifugation at 8000rpm for 1 minute. After centrifugation the collection tubes were discarded and new columns were placed into the new collection tubes. To this 500 µl AW1 was added and then again centrifuged at 8000rpm for 1 minute duration. This process was repeated by changing only the buffer with 500 µl AW2. Column was spinned at 13200rpm for 3 minutes in order to dry the column membrane. In continuation, 50 µl AE buffer was added into the column and then incubated for 5 minutes. Final centrifugation was done at 13200 rpm for 3 minutes to collect the elution buffer which contained DNA. The quality and quantity of gDNA were verified by Nanodrop ND-2000. DNA concentration (ng/µl) and DNA purity (A260/A280) were calculated using the spectrophotometer (NanoDrop ND-2000) according to the manufacturer's instructions.

APPENDIX B: DETAIL OF RNEASY MINI KIT EXTRACTION (QIAGEN GMBH GERMANY) PROTOCOL

Fresh-frozen tissue sections were incubated in 350 µl Buffer RLT which contained 3.5 µl beta mercaptoethanol. The mixture was homogenized with 1 ml syringe. One volume of 70% ethanol was added into the mixture followed by vortexing for 30 seconds. The mixture was transferred into RNeasy MinElute spin column assembly with 2 ml collection tube and centrifuge for 15 second at 10,000 rpm. The flow through solution was discarded. There were 350 µl of buffer RW1 was added into the RNeasy MinElute spin column assembly with 2 ml collection tubes and centrifuged and for 15 seconds at 10,000 rpm to wash the spin column. The flow through solution and collection tubes was discarded. RNeasy MinElute spin column was placed with a new 2 ml collection tube and 500 µl Buffer RPE was added to the spin column continued by centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. Total of 500 µl of 80% ethanol was added to the RNeasy MinElute spin column and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow through solution and collection tube was discarded. RNeasy MinElute spin column was placed with a new 2 ml collection tube. The lid of the spin column was opened during centrifugation at full speed for 5 minutes. The flow through solution and collection tube was discarded. The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube. Total of 14 µl of RNase free water was directly added to the center of the spin column membrane and centrifuged for 3 minutes at full speed to elute the RNA. The RNA integrity number (RIN) and concentration of RNA extracted was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies).

APPENDIX C: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE 75 PATIENTS FOR ARRAY CGH STUDY

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOUR SUBSITE	TUMOUR SIZE	LNM	TUMOUR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS
1	04-0014-11	MALE	78	CHINESE	NO	YES	NO	GUM	T1	NO	I	EARLY	MODERATE	DEAD	15
2	04-0020-11	FEMALE	77	INDIAN	YES	NO	NO	BM	T2	NO	II	EARLY	WELL	ALIVE	21
3	04-0028-11	MALE	65	CHINESE	YES	YES	NO	FOM	T3	YES	III	ADVANCED	MODERATE	DEAD	6
4	04-0030-11	MALE	66	CHINESE	YES	NO	NO	OTHER	T2	YES	III	ADVANCED	WELL	DEAD	21
5	04-0034-10	FEMALE	70	INDIAN	NO	NO	NO	TONGUE	T2	NO	II	EARLY	WELL	DEAD	29
6	04-0002-10	MALE	66	MALAY	NO	NO	NO	GUM	T4	YES	IV	ADVANCED	WELL	DEAD	12
7	04-0005-09	FEMALE	59	INDIAN	NO	YES	YES	BM	T2	NO	II	EARLY	WELL	ALIVE	58
8	04-0006-09	MALE	55	INDIAN	YES	NO	NO	TONGUE	T2	NO	II	EARLY	WELL	DEAD	42
9	04-0007-12	FEMALE	33	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	ALIVE	22
10	04-0008-10	MALE	62	MALAY	YES	NO	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	ALIVE	44
11	04-0013-09	MALE	63	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	MODERATE	ALIVE	55
12	04-0013-11	FEMALE	65	INDIAN	NO	NO	YES	BM+GUM	T2	YES	III	ADVANCED	WELL	ALIVE	43
13	04-0015-09	FEMALE	60	INDIAN	NO	YES	YES		T1	NO	I	EARLY	MODERATE	ALIVE	54
14	04-0040-10	FEMALE	79	INDIAN	NO	NO	YES	OTHER	T4	NO	IV	ADVANCED	WELL	DEAD	19
15	06-0002-13	FEMALE	65	INDIAN	NO	NO	YES	BM	T3	YES	IV	ADVANCED	POOR	DEAD	7
16	06-0012-13	MALE	43	CHINESE	NO	NO	NO	TONGUE	T3	YES	IV	ADVANCED	MODERATE	DEAD	7
17	06-0074-12	FEMALE	60	INDIAN	NO	NO	YES	BM	T1	YES	IV	ADVANCED	MODERATE	DEAD	15
18	06-0002-11	FEMALE	75	INDIAN	NO	NO	YES	BM	T4	YES	IV	ADVANCED	MODERATE	DEAD	1
19	06-0007-11	FEMALE	51	CHINESE	NO	NO	NO	TONGUE	T1	YES	IV	ADVANCED	WELL	ALIVE	36
20	06-0009-11	FEMALE	42	INDIAN	NO	NO	YES	BM	T3	YES	IV	ADVANCED	MODERATE	DEAD	9
21	06-0011-11	MALE	49	INDIAN	YES	YES	YES	FOM+TONG	T4	YES	IV	ADVANCED	MODERATE	DEAD	8
22	06-0014-11	MALE	66	INDIAN	NO	NO	YES	HP	T2	NO	II	EARLY	MODERATE	ALIVE	33
23	06-0018-10	MALE	56	INDIAN	YES	YES	NO	T+FOM	T2	YES	IV	ADVANCED	MODERATE	ALIVE	43
24	06-0019-13	MALE	56	INDIAN	NO	NO	NO	PALATE	T2	YES	IV	ADVANCED	MODERATE	DEAD	12

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOUR SUBSITE	TUMOUR SIZE	LNM	TUMOUR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS
25	06-0020-12	MALE	73	INDIAN	NO	NO	YES	BM	T2	YES	IV	ADVANCED	MODERATE	ALIVE	23
26	06-0021-11	FEMALE	48	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	ALIVE	33
27	06-0022-12	FEMALE	35	INDIAN	YES	YES	NO	TONGUE	T1	NO	I	EARLY	POOR	ALIVE	22
28	06-0028-11	FEMALE	71	INDIAN	NO	NO	YES	GUM+BM	T2	NO	II	EARLY	MODERATE	ALIVE	30
29	06-0031-11	FEMALE	64	INDIAN	NO	NO	YES	BM+HP	T3	NO	III	ADVANCED	MODERATE	DEAD	6
30	06-0033-10	FEMALE	72	INDIAN	NO	NO	YES	GUM	T3	YES	IV	ADVANCED	MODERATE	DEAD	6
31	06-0036-11	FEMALE	62	INDIAN	NO	NO	YES	BM	T2	YES	IV	ADVANCED	MODERATE	DEAD	9
32	06-0038-11	FEMALE	58	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	ALIVE	36
33	06-0040-09	FEMALE	74	INDIAN	NO	NO	YES	BM+HP	T4	YES	IV	ADVANCED	WELL	DEAD	1
34	06-0044-10	FEMALE	49	INDIAN	NO	NO	YES	BM	T1	YES	III	ADVANCED	MODERATE	ALIVE	44
35	06-0050-10	FEMALE	52	MALAY	NO	NO	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	DEAD	13
36	06-0037-08	FEMALE	36	INDIAN	NO	NO	YES	BM	T1	NO	I	EARLY	WELL	ALIVE	65
37	01-0049-12	FEMALE	69	INDIAN	NO	NO	YES		T2	NO	II	EARLY	MODERATE	ALIVE	14
38	01-0001-06	FEMALE	71	INDIAN	NO	NO	YES	BM+GUM	T3	YES	IV	ADVANCED	WELL	DEAD	8
39	01-0003-10	FEMALE	57	INDIAN	NO	NO	YES	LIP	T2	NO	II	EARLY	WELL	ALIVE	48
40	01-0005-12	FEMALE	49	MALAY	NO	NO	NO	GUM+BM	T2	YES	IV	ADVANCED	MODERATE	DEAD	1
41	01-0006-09	MALE	52	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	WELL	ALIVE	57
42	01-0009-11	FEMALE	62	MALAY	NO	NO	NO	BM	T3	YES	IV	ADVANCED	WELL	ALIVE	34
43	01-0010-10	FEMALE	56	CHINESE	NO	NO	NO	TONGUE	T3	NO	III	ADVANCED	WELL	DEAD	14
44	01-0011-10	FEMALE	63	INDIAN	NO	NO	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	DEAD	12
45	01-0012-11	MALE	57	CHINESE	NO	NO	NO	GUM	T1	NO	I	EARLY	WELL	ALIVE	34
46	01-0018-11	MALE	69	CHINESE	NO	NO	NO	GUM	T4	NO	IV	ADVANCED	WELL	DEAD	12
47	01-0019-10	FEMALE	76	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	ALIVE	48
48	01-0020-11	FEMALE	56	INDIAN	NO	NO	YES	GUM	T1	YES	III	ADVANCED	WELL	ALIVE	32
49	01-0024-10	FEMALE	29	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	POOR	ALIVE	42

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOUR SUBSITE	TUMOUR SIZE	LNM	TUMOUR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS
50	01-0025-12	MALE	62	CHINESE	YES	YES	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	ALIVE	21
51	01-0026-06	FEMALE	58	INDIAN	NO	NO	YES	BM+GUM	T1	NO	I	EARLY	WELL	ALIVE	89
52	01-0030-10	FEMALE	33	MALAY	NO	NO	NO	TONGUE	T3	YES	IV	ADVANCED	MODERATE	DEAD	15
53	01-0032-12	MALE	69	CHINESE	YES	YES	NO	HP	T4	NO	IV	ADVANCED	MODERATE	ALIVE	19
54	01-0036-11	FEMALE	69	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	ALIVE	28
55	01-0048-11	MALE	87	INDIAN	YES	NO	NO	TONGUE	T2	YES	IV	ADVANCED	WELL	DEAD	11
56	11-0007-10	FEMALE	50	OTHER	NO	NO	YES	BM	T2	NO	II	EARLY	WELL	ALIVE	43
57	11-0008-10	FEMALE	56	OTHER	YES	NO	NO	BM+GUM	T4	YES	IV	ADVANCED	WELL	DEAD	12
58	11-0018-10	MALE	57	OTHER	YES	YES	NO	GUM+BM+F	T4	YES	IV	ADVANCED	WELL	DEAD	7
59	11-0001-12	FEMALE	27	CHINESE	NO	NO	NO	TONGUE	T2	YES	IV	ADVANCED	POOR	DEAD	3
60	11-0002-08	FEMALE	71	OTHER	NO	NO	YES	LIP	T2	NO	II	EARLY	WELL	DEAD	2
61	11-0002-12	FEMALE	66	OTHER	YES	NO	NO	GUM+OP	T4	YES	IV	ADVANCED	WELL	ALIVE	25
62	11-0004-07	FEMALE	58	OTHER	NO	NO	YES	SP+TONGU	T3	NO	III	ADVANCED	MODERATE	DEAD	11
63	11-0004-12	MALE	81	CHINESE	YES	NO	NO	GUM	T4	NO	IV	ADVANCED	WELL	ALIVE	24
64	11-0005-07	MALE	30	CHINESE	NO	NO	NO	TONGUE	T3	YES	III	ADVANCED	WELL	DEAD	12
65	11-0006-08	FEMALE	77	OTHER	YES	NO	YES	PALATE+T	T4	NO	IV	ADVANCED	MODERATE	DEAD	3
66	11-0007-04	MALE	69	OTHER	YES	NO	YES	TONGUE	T2	NO	II	EARLY	WELL	ALIVE	114
67	11-0008-04	FEMALE	54	OTHER	YES	NO	YES	GUM	T2	YES	IV	ADVANCED	MODERATE	DEAD	12
68	11-0009-07	FEMALE	51	MALAY	NO	NO	NO	TONGUE	T3	NO	III	ADVANCED	WELL	DEAD	10
69	11-0009-10	FEMALE	27	CHINESE	NO	NO	NO	TONGUE	T1	YES	III	ADVANCED	WELL	ALIVE	44
70	11-0013-10	MALE	58	OTHER	YES	YES	NO	FOM	T4	NO	IV	ADVANCED	MODERATE	ALIVE	41
71	11-0014-10	FEMALE	69	CHINESE	NO	NO	NO	GUM	T4	YES	IV	ADVANCED	MODERATE	ALIVE	47
72	11-0015-10	MALE	59	MALAY	YES	NO	NO	BM	T3	NO	III	ADVANCED	MODERATE	ALIVE	39
73	11-0017-10	FEMALE	67	MALAY	NO	NO	YES	GUM	T4	NO	III	ADVANCED	MODERATE	DEAD	9

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOUR SUBSITE	TUMOUR SIZE	LNM	TUMOUR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS
74	11-0049-11	MALE	37	OTHER	NO	NO	NO	GUM	T3	YES	IV	ADVANCED	WELL	ALIVE	28
75	11-0010-10	FEMALE	35	CHINESE	NO	NO	NO	TONGUE	T1	NO	I	EARLY	WELL	ALIVE	43

APPENDIX D: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE 66 PATIENTS FOR TAQMAN PCR ASSAY

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZE	LNM	TUMOR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS	LRP12	CCND1	TPM2	FSCN1	EGFR	CLPTM1L	CHL1	CSMD1
1	04-0014-11	MALE	78	CHINESE	NO	YES	NO	GUM	T1	NO	I	EARLY	MODERATE	dead	15	6.07	10.01	1.85	2.11	10.81	2.39	1.7	1.74
2	04-0028-11	MALE	65	CHINESE	YES	YES	NO	FOM	T3	YES	III	ADVANCED	MODERATE	dead	6	1.74	1.25	1.5	2.02	2.24	1.76	1.81	1.56
3	04-0034-10	FEMALE	70	INDIAN	NO	NO	NO	TONGUE	T2	NO	II	EARLY	WELL	dead	29	1.49	1.53	1.57	2.29	2.4	2.17	0.98	1.21
4	04-0002-10	MALE	66	MALAY	NO	NO	NO	GUM	T4	YES	IV	ADVANCED	WELL	dead	12	1.4	4.46	4.66	3.09	228.36	1.47	0.79	0.72
5	04-0006-09	MALE	55	INDIAN	YES	NO	NO	TONGUE	T2	NO	II	EARLY	WELL	dead	42	0.42	0.26	0.25	0.48	0.33	0.44	0.26	0.32
6	04-0007-12	FEMALE	33	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	alive	22	2.34	2.17	1.97	1.53	1.88	2.9	1.42	1.89
7	04-0008-10	MALE	62	MALAY	YES	NO	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	alive	44	0.39	0.2	0.19	0.26	0.41	0.28	0.24	0.18
8	04-0013-09	MALE	63	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	MODERATE	alive	55	0.32	0.27	0.22	0.37	0.3	0.38	0.24	0.28
9	04-0040-10	FEMALE	79	INDIAN	NO	NO	YES	OTHER	T4	NO	IV	ADVANCED	WELL	dead	19	0.89	0.33	0.28	0.57	0.37	0.37	0.34	0.36
10	06-0002-13	FEMALE	65	INDIAN	NO	NO	YES	BM	T3	YES	IV	ADVANCED	POOR	dead	7	1.59	1.68	1.81	1.88	33.34	2.4	1.18	0.9
11	06-0012-13	MALE	43	CHINESE	NO	NO	NO	TONGUE	T3	YES	IV	ADVANCED	MODERATE	dead	7	1.91	2.93	2.04	2.09	14.72	2.8	1.39	1.44
12	06-0074-12	FEMALE	60	INDIAN	NO	NO	YES	BM	T1	YES	IV	ADVANCED	MODERATE	dead	15	0.8	1.09	1.08	1.34	1.61	1.76	0.9	1.28
13	06-0002-11	FEMALE	75	INDIAN	NO	NO	YES	BM	T4	YES	IV	ADVANCED	MODERATE	dead	1	0.75	0.27	0.38	0.54	0.33	0.37	0.19	0.08
14	06-0009-11	FEMALE	42	INDIAN	NO	NO	YES	BM	T3	YES	IV	ADVANCED	MODERATE	dead	9	0.54	0.75	0.13	0.32	1.86	0.27	0.17	0.1
15	06-0011-11	MALE	49	INDIAN	YES	YES	YES	FOM+TONG	T4	YES	IV	ADVANCED	MODERATE	dead	8	2.01	1.88	1.45	1.53	1.76	2.54	2.73	1.61
16	06-0014-11	MALE	66	INDIAN	NO	NO	YES	HP	T2	NO	II	EARLY	MODERATE	alive	33	1.21	1.61	2.18	22.94	2.1	2.68	1.42	34.48
17	06-0018-10	MALE	56	INDIAN	YES	YES	NO	T+FOM	T2	YES	IV	ADVANCED	MODERATE	alive	43	2.22	1.86	1.95	2.33	2.37	3.17	5.92	2.8
18	06-0019-13	MALE	56	INDIAN	NO	NO	NO	PALATE	T2	YES	IV	ADVANCED	MODERATE	dead	12	2.05	1.66	1.77	1.75	13.48	2.33	1.51	1.59
19	06-0020-12	MALE	73	INDIAN	NO	NO	YES	BM	T2	YES	IV	ADVANCED	MODERATE	alive	23	1.43	1.6	2.7	24.51	2.67	3.46	1.51	31.35
20	06-0021-11	FEMALE	48	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	alive	33	3.45	10.9	2.26	3.2	4.78	3.02	5.73	2.08
21	06-0022-12	FEMALE	35	INDIAN	YES	YES	NO	TONGUE	T1	NO	I	EARLY	POOR	alive	22	2.47	1.78	1.99	3.01	3.24	21.52	2.23	1.64
22	06-0028-11	FEMALE	71	INDIAN	NO	NO	YES	GUM+BM	T2	NO	II	EARLY	MODERATE	alive	30	1.94	2.15	2.36	3.47	2.71	36.66	2.58	2.06
23	06-0031-11	FEMALE	64	INDIAN	NO	NO	YES	BM+HP	T3	NO	III	ADVANCED	MODERATE	dead	6	2.06	2.18	2.5	2.95	2.03	21.89	1.77	1.23
24	06-0033-10	FEMALE	72	INDIAN	NO	NO	YES	GUM	T3	YES	IV	ADVANCED	MODERATE	dead	6	1.61	1.78	1.56	3.16	2.74	2.46	1.73	1.8
25	06-0036-11	FEMALE	62	INDIAN	NO	NO	YES	BM	T2	YES	IV	ADVANCED	MODERATE	dead	9	1.25	13.48	1.71	2.5	6.73	1.91	2.04	1.11

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZE	LNM	TUMOR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS	LRP12	CCND1	TPM2	FSCN1	EGFR	CLPTM1L	CHL1	CSMD1
26	06-0038-11	FEMALE	58	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	alive	36	0.77	1.83	1.74	2.39	1.64	1.76	4.74	1.73
27	06-0040-09	FEMALE	74	INDIAN	NO	NO	YES	BM+HP	T4	YES	IV	ADVANCED	WELL	dead	1	1.43	1.58	1.64	3.83	2.62	2.33	1.96	2.6
28	06-0044-10	FEMALE	49	INDIAN	NO	NO	YES	BM	T1	YES	III	ADVANCED	MODERATE	alive	44	2.08	2.2	2.19	2.31	2.31	2.54	1.56	1.68
29	06-0037-08	FEMALE	36	INDIAN	NO	NO	YES	BM	T1	NO	I	EARLY	WELL	alive	65	1.26	1.85	1.67	2.59	1.9	2.4	1.45	2.02
30	01-0049-12	FEMALE	69	INDIAN	NO	NO	YES		T2	NO	II	EARLY	MODERATE	alive	14	3.55	3.86	3.96	4.34	3.55	4.03	2.14	4.92
31	01-0001-06	FEMALE	71	INDIAN	NO	NO	YES	BM+GUM	T3	YES	IV	ADVANCED	WELL	dead	8	2.59	2.02	1.71	1.67	3.36	16.26	2.45	15
32	01-0003-10	FEMALE	57	INDIAN	NO	NO	YES	LIP	T2	NO	II	EARLY	WELL	alive	48	1.41	1.75	1.65	1.5	1.86	2.34	4.16	1.6
33	01-0005-12	FEMALE	49	MALAY	NO	NO	NO	GUM+BM	T2	YES	IV	ADVANCED	MODERATE	dead	1	5.63	4.37	2.98	1.87	5.87	12.42	4.34	13.9
34	01-0006-09	MALE	52	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	WELL	alive	57	4.27	2.16	2.12	2.21	3.24	19.94	4.71	20.21
35	01-0009-11	FEMALE	62	MALAY	NO	NO	NO	BM	T3	YES	IV	ADVANCED	WELL	alive	34	1.72	10.2	1.44	3.07	2.9	23.94	1.24	2.33
36	01-0010-10	FEMALE	56	CHINESE	NO	NO	NO	TONGUE	T3	NO	III	ADVANCED	WELL	dead	14	2.25	7.73	1.07	1.81	4.36	16.61	1.33	1.67
37	01-0011-10	FEMALE	63	INDIAN	NO	NO	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	dead	12	1.78	9.92	1.46	1.79	3	15.67	1.71	2.76
38	01-0012-11	MALE	57	CHINESE	NO	NO	NO	GUM	T1	NO	I	EARLY	WELL	alive	34	5.2	13.06	2.11	2.89	13.21	2.76	1.47	1.17
39	01-0019-10	FEMALE	76	INDIAN	NO	NO	YES	BM	T2	NO	II	EARLY	MODERATE	alive	48	1.05	1.46	1.29	2.2	2.02	1.91	0.99	1.4
40	01-0020-11	FEMALE	56	INDIAN	NO	NO	YES	GUM	T1	YES	III	ADVANCED	WELL	alive	32	3.93	3.28	2.69	2.64	3.45	4.36	10.87	3.98
41	01-0024-10	FEMALE	29	CHINESE	YES	NO	NO	TONGUE	T1	NO	I	EARLY	POOR	alive	42	1.86	1.44	1.03	1.95	1.57	2.04	3.77	1.32
42	01-0025-12	MALE	62	CHINESE	YES	YES	NO	TONGUE	T2	YES	IV	ADVANCED	MODERATE	alive	21	2.28	1.62	1.39	2.85	2.15	2.67	6.78	1.91
43	01-0026-06	FEMALE	58	INDIAN	NO	NO	YES	BM+GUM	T1	NO	I	EARLY	WELL	alive	89	1.26	1.92	2.4	34.74	3.88	2.68	1.79	15.19
44	01-0030-10	FEMALE	33	MALAY	NO	NO	NO	TONGUE	T3	YES	IV	ADVANCED	MODERATE	dead	15	1.97	1.56	1.34	2.15	2.01	2.04	7.26	1.66
45	01-0032-12	MALE	69	CHINESE	YES	YES	NO	HP	T4	NO	IV	ADVANCED	MODERATE	alive	19	1.58	2.76	1.76	2.27	2.42	2.45	1.13	3.05
46	01-0048-11	MALE	87	INDIAN	YES	NO	NO	tongue	T2	YES	IV	ADVANCED	WELL	dead	11	0.77	1.69	4.79	1.7	7.78	1.11	0.87	0.71
47	11-0007-10	FEMALE	50	OTHER	NO	NO	YES	BM	T2	NO	II	EARLY	WELL	alive	43	1.39	2.36	9.12	2.14	1.8	2.04	2.32	2.29E+08
48	11-0008-10	FEMALE	56	OTHER	YES	NO	NO	BM+GUM	T4	YES	IV	ADVANCED	WELL	dead	12	1.67	2.09	2.39	3.65	2.75	1.81	3.32	2.52
49	11-0018-10	MALE	57	OTHER	YES	YES	NO	GUM+BM+F	T4	YES	IV	ADVANCED	WELL	dead	7	2.15	3.59	1.67	2.54	2.29	2.14	1.19	1.04
50	11-0001-12	FEMALE	27	CHINESE	NO	NO	NO	TONGUE	T2	YES	IV	ADVANCED	POOR	dead	3	0.2	0.66	0.19	0.15	0.29	0.15	0.04	0.1

NO	IRPA	GENDER	AGE	ETHNICITY	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZE	LNM	TUMOR STAGE	EARLY/ ADVANCED	DIFFERENTIATION	STATUS	MONTHS	LRP12	CCND1	TPM2	FSCN1	EGFR	CLPTM1L	CHL1	CSMD1
51	11-0002-08	FEMALE	71	OTHER	NO	NO	YES	LIP	T2	NO	II	EARLY	WELL	dead	2	2.03	1.98	2.25	2.81	3.24	2.12	1.56	1.53
52	11-0002-12	FEMALE	66	OTHER	YES	NO	NO	GUM+OP	T4	YES	IV	ADVANCED	WELL	alive	25	0.52	0.65	0.4	0.38	0.65	0.55	0.25	0.51
53	11-0004-07	FEMALE	58	OTHER	NO	NO	YES	SP+TONGU	T3	NO	III	ADVANCED	MODERATE	dead	11	3.71	1.31	1.38	2.34	1.94	2	1.03	1.5
54	11-0004-12	MALE	81	CHINESE	YES	NO	NO	GUM	T4	NO	IV	ADVANCED	WELL	alive	24	0.63	0.43	0.22	0.24	0.38	0.23	0.14	0.29
55	11-0005-07	MALE	30	CHINESE	NO	NO	NO	TONGUE	T3	YES	III	ADVANCED	WELL	dead	12	6.45	1.52	1.07	1.34	4.38	1.69	1.2	1.22
56	11-0006-08	FEMALE	77	OTHER	YES	NO	YES	PALATE+T	T4	NO	IV	ADVANCED	MODERATE	dead	3	2.83	2.99	14.07	3.05	3.33	2.9	4.38	2.56E+08
57	11-0007-04	MALE	69	OTHER	YES	NO	YES	TONGUE	T2	NO	II	EARLY	WELL	alive	114	7.3	11.2	1.49	2.69	16.11	3.29	1.6	1.24
58	11-0008-04	FEMALE	54	OTHER	YES	NO	YES	GUM	T2	YES	IV	ADVANCED	MODERATE	dead	12	3.32	0.76	0.94	0.75	5.51	1.04	0.7	0.72
59	11-0009-07	FEMALE	51	MALAY	NO	NO	NO	TONGUE	T3	NO	III	ADVANCED	WELL	dead	10	1.09	1.59	6.54	1.78	1.51	1.66	2.64	1.35E+09
60	11-0009-10	FEMALE	27	CHINESE	NO	NO	NO	TONGUE	T1	YES	III	ADVANCED	WELL	alive	44	1.46	2.31	2.25	2.48	2.28	1.97	2.17	2.09
61	11-0013-10	MALE	58	OTHER	YES	YES	NO	FOM	T4	NO	IV	ADVANCED	MODERATE	alive	41	2.12	2.45	1.79	3.48	3.24	2.55	2.64	1.65
62	11-0014-10	FEMALE	69	CHINESE	NO	NO	NO	GUM	T4	YES	IV	ADVANCED	MODERATE	alive	47	2.54	1.86	1.92	1.72	2.49	1.65	3.67	2.45
63	11-0015-10	MALE	59	MALAY	YES	NO	NO	BM	T3	NO	III	ADVANCED	MODERATE	alive	39	2.38	2.06	1.46	2.46	23.49	2.71	2.27	1.62
64	11-0017-10	FEMALE	67	MALAY	NO	NO	YES	GUM	T4	NO	III	ADVANCED	MODERATE	dead	9	1.11	1.88	1.62	1.47	3.27	1.99	3.54	1.28
65	11-0049-11	MALE	37	OTHER	NO	NO	NO	GUM	T3	YES	IV	ADVANCED	WELL	alive	28	2.07	1.78	2.13	1.24	1.67	1.81	1.23	1.79
66	11-0010-10	FEMALE	35	CHINESE	NO	NO	NO	TONGUE	T1	NO	I	EARLY	WELL	alive	43	1.33	2.06	1.97	2.51	1.7	1.89	2.85	1.89

APPENDIX E: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE 68 PATIENTS FOR MMP13 GENE EXPRESSION QPCR STUDY

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	RQ MMP13	ALIVE/ DEATH	MONTHS
1	01-0023-09-1	Male	42	YES	NO	NO	Tongue	No	Early	137.61	ALIVE	22
2	01-0005-09-1	Female	71	NO	NO	NO	Non tongue	No	Advanced	228.85	ALIVE	24
3	06-0029-08-1	Female	86	YES	NO	NO	Non tongue	Yes	Advanced	6.22	DECEASED	8
4	06-0012-08-1	Male	47	YES	NO	NO	Tongue	No	Early	121.93	ALIVE	39
5	01-0048-07-1	Male	71	YES	NO	NO	Tongue	Yes	Advanced	1538.79	DECEASED	5
6	04-0021-08-1	Female	45	NO	NO	NO	Tongue	No	Early	503.97	LOF	6
7	01-0006-09-1	Male	52	YES	NO	NO	Tongue	No	Early	23.14	ALIVE	31
8	04-0017-09-1	Male	30	NO	NO	NO	Tongue	Yes	Advanced	228.64	DECEASED	13
9	11-0005-07-1	Male	34	YES	NO	NO	Tongue	Yes	Advanced	944.77	DECEASED	13
10	04-0021-06-1	Male	48	YES	NO	NO	Tongue	Yes	Advanced	492.31	DECEASED	1
11	01-0004-09-1	Female	75	NO	NO	YES	Non tongue	No	Advanced	259.8	ALIVE	14
12	11-0039-05-1	Female	52	NO	NO	YES	Tongue	Yes	Advanced	1139.5	DECEASED	1
13	01-0046-05-1	Male	45	NO	YES	NO	Tongue	Yes	Advanced	46.51	DECEASED	9
14	01-0036-08-1	Female	63	NO	YES	YES	Non tongue	Yes	Advanced	145.68	ALIVE	42
15	04-0014-09-1	Female	37	NO	NO	NO	Tongue	No	Early	207.54	LOF	15
16	01-0028-09-1	Female	44	YES	YES	NO	Tongue	Yes	Advanced	73.95	DECEASED	10
17	04-0006-10-1	Male	36	YES	YES	NO	Tongue	No	Early	945.99	ALIVE	12
18	01-0002-10-1	Male	72	NO	NO	YES	Non tongue	No	Advanced	5.53	LOF	9
19	06-0005-10-1	Female	63	NO	YES	YES	Non tongue	No	Early	2.47	DECEASED	3
20	06-0027-09-1	Male	62	NO	YES	YES	Non tongue	Yes	Advanced	34.65	DECEASED	4
21	01-0022-07-1	Male	49	YES	NO	NO	Non tongue	No	Advanced	26.12	LOF	39
22	01-0024-08-1	Female	61	NO	NO	YES	Non tongue	Yes	Advanced	207.28	ALIVE	33
23	01-0007-09-1	Female	61	NO	NO	YES	Non tongue	Yes	Advanced	83.29	LOF	3
24	06-0030-10-1	Female	53	NO	NO	YES	Tongue	Yes	Advanced	4.24	DECEASED	8

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	RQ MMP13	ALIVE/ DEATH	MONTHS
25	04-0032-10-1	Female	86	NO	NO	YES	Tongue	No	Early	57.39	ALIVE	18
26	04-0019-05-1	Female	55	NO	NO	YES	Non tongue	No	Early	1.56	LOF	52
27	04-0014-08-1	Male	60	YES	NO	NO	Non tongue	Yes	Advanced	2.3	ALIVE	40
28	04-0013-08-1	Female	62	NO	NO	YES	Tongue	Yes	Advanced	8.07	LOF	6
29	11-0002-08	Female	71	NO	NO	YES	Non tongue	No	Early	53.6	DECEASED	12
30	01-0058-07	Female	66	YES	YES	NO	Non tongue	No	Early	4	ALIVE	35
31	01-0107-08	Female	65	YES	NO	NO	Non tongue	No	Advanced	33.76	DECEASED	15
32	01-0011-10	Female	63	NO	NO	NO	Tongue	Yes	Advanced	8.15	DECEASED	6
33	01-0106-08	Male	20	NO	YES	NO	Non tongue	Yes	Advanced	11.84	DECEASED	12
34	01-0022-10	Female	51	NO	NO	NO	Non tongue	Yes	Advanced	383.2	DECEASED	9
35	01-0024-10	Male	29	YES	NO	NO	Tongue	No	Early	6.49	ALIVE	14
36	01-0025-12	Male	62	YES	YES	NO	Tongue	Yes	Advanced	119.17	ALIVE	7
37	01-0005-12	Female	49	NO	NO	NO	Non tongue	Yes	Advanced	23.34	DECEASED	1
38	01-0019-10	Female	76	NO	NO	YES	Non tongue	No	Early	4.55	ALIVE	24
39	01-0012-11	Female	57	NO	NO	NO	Non tongue	No	Early	162.53	ALIVE	17
40	06-0012-08	Male	47	YES	NO	NO	Tongue	No	Early	126.17	ALIVE	39
41	06-0010-09	Female	73	NO	YES	YES	Tongue	Yes	Advanced	0.49	LOF	5
42	06-0032-08	Male	48	YES	NO	YES	Non tongue	Yes	Advanced	1.69	DECEASED	8
43	06-0033-10	Female	72	NO	NO	YES	Non tongue	Yes	Advanced	568.4	DECEASED	6
44	06-0005-09	Female	49	NO	NO	YES	Tongue	No	Early	956.81	ALIVE	27
45	06-0018-10	Male	56	YES	YES	NO	Tongue	Yes	Advanced	30.7	ALIVE	31
46	06-0055-10	Female	60	NO	YES	YES	Non tongue	No	Early	501.07	ALIVE	25
47	06-0002-11	Female	75	NO	NO	YES	Non tongue	Yes	Advanced	3.56	DECEASED	1
48	06-0011-11	Male	48	YES	YES	YES	Tongue	Yes	Advanced	1517.47	DECEASED	11

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	RQ MMP13	ALIVE/ DEATH	MONTHS
49	06-0028-11	Female	71	NO	NO	YES	Non tongue	No	Early	0.49	ALIVE	18
50	06-0009-11	Female	41	NO	NO	YES	Non tongue	Yes	Advanced	697.69	DECEASED	9
51	06-0007-11	Female	51	NO	NO	NO	Tongue	Yes	Advanced	42.03	ALIVE	12
52	06-0031-11	Female	64	NO	YES	YES	Non tongue	Yes	Advanced	239.01	DECEASED	2
53	04-0007-08	Female	52	NO	NO	NO	Tongue	No	Early	9.98	ALIVE	48
54	04-0022-09	Female	70	NO	NO	NO	Non tongue	No	Advanced	0.38	ALIVE	12
55	04-0040-10	Female	79	NO	NO	YES	Non tongue	No	Early	60.98	DECEASED	18
56	04-0005-09	Female	59	NO	YES	YES	Non tongue	No	Early	243.4	ALIVE	33
57	04-0026-08	Female	29	NO	NO	NO	Tongue	Yes	Advanced	4.71	DECEASED	32
58	04-0008-10	Male	62	YES	NO	NO	Tongue	Yes	Advanced	4.22	ALIVE	33
59	04-0013-11	Female	65	NO	NO	YES	Non tongue	Yes	Advanced	12.8	ALIVE	19
60	04-0002-10	Male	66	NO	NO	NO	Non tongue	Yes	Advanced	349.87	DECEASED	12
61	11-0004-07	Female	58	NO	NO	YES	Tongue	No	Advanced	1759.66	DECEASED	11
62	11-0010-10	Female	35	NO	NO	NO	Tongue	No	Early	275.33	ALIVE	30
63	11-0013-10	Male	58	YES	YES	NO	Non tongue	No	Advanced	31.51	ALIVE	28
64	11-0015-10	Female	59	YES	NO	NO	Non tongue	No	Advanced	19.61	ALIVE	26
65	11-0018-10	Female	46	YES	YES	NO	Non tongue	Yes	Advanced	2239.82	DECEASED	29
66	01-0009-11	Female	61	NO	NO	NO	Non tongue	Yes	Advanced	596.09	DECEASED	12
67	01-0018-11	Male	69	NO	NO	NO	Non tongue	No	Advanced	152.2	ALIVE	.
68	04-0008-09	Female	.	YES	NO	NO	Non tongue	No	Advanced	52.64	ALIVE	.

APPENDIX F: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE 68 PATIENTS FOR ISG15 GENE EXPRESSION QPCR STUDY

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	ALIVE/ DEATH	MONTHS	RQ ISG15
1	01-0023-09-1	Male	42	YES	NO	NO	Tongue	No	Early	ALIVE	22	18.52
2	06-0012-08-1	Male	47	YES	NO	NO	Tongue	No	Early	ALIVE	39	19.12
3	01-0048-07-1	Male	71	YES	NO	NO	Tongue		Advanced	DECEASED	5	271.09
4	04-0021-08-1	Female	45	NO	NO	NO	Tongue	No	Early	LOF	6	2.5
5	01-0006-09-1	Male	52	YES	NO	NO	Tongue	No	Early	ALIVE	31	26.14
6	04-0017-09-1	Male	30	NO	NO	NO	Tongue	Yes	Advanced	DECEASED	13	85.7
7	11-0005-07-1	Male	34	YES	NO	NO	Tongue	Yes	Advanced	DECEASED	13	1.44
8	04-0021-06-1	Male	48	YES	NO	NO	Tongue	Yes	Advanced	DECEASED	1	36.21
9	11-0039-05-1	Female	52	NO	NO	YES	Tongue	Yes	Advanced	DECEASED	1	52.32
10	01-0046-05-1	Male	45	NO	YES	NO	Tongue	Yes	Advanced	DECEASED	9	9.1
11	04-0014-09-1	Female	37	NO	NO	NO	Tongue	No	Early	LOF	15	7.98
12	01-0028-09-1	Female	44	YES	YES	NO	Tongue	Yes	Advanced	DECEASED	10	13.79
13	04-0006-10-1	Male	36	YES	YES	NO	Tongue	No	Early	ALIVE	12	6.82
14	06-0030-10-1	Female	53	NO	NO	YES	Tongue	Yes	Advanced	DECEASED	8	1.45
15	04-0032-10-1	Female	86	NO	NO	YES	Tongue	No	Early	ALIVE	18	54.44
16	04-0013-08-1	Female	62	NO	NO	YES	Tongue	Yes	Advanced	LOF	6	371.93
17	01-0011-10	Female	63	NO	NO	NO	Tongue	Yes	Advanced	DECEASED	6	65.35
18	01-0024-10	Male	29	YES	NO	NO	Tongue	No	Early	ALIVE	14	23.93
19	01-0025-12	Male	62	YES	YES	NO	Tongue	Yes	Advanced	ALIVE	7	4.55
20	06-0012-08	Male	47	YES	NO	NO	Tongue	No	Early	ALIVE	39	23.13
21	06-0010-09	Female	73	NO	YES	YES	Tongue	Yes	Advanced	LOF	5	5.4
22	06-0005-09	Female	49	NO	NO	YES	Tongue	No	Early	ALIVE	27	101.77

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	ALIVE/ DEATH	MONTHS	RQ ISG15
23	06-0018-10	Male	56	YES	YES	NO	Tongue	Yes	Advanced	ALIVE	31	37.06
24	06-0011-11	Male	48	YES	YES	YES	Tongue	Yes	Advanced	DECEASED	11	28.36
25	06-0007-11	Female	51	NO	NO	NO	Tongue	Yes	Advanced	ALIVE	12	223.75
26	04-0007-08	Female	52	NO	NO	NO	Tongue	No	Early	ALIVE	48	3.05
27	04-0026-08	Female	29	NO	NO	NO	Tongue	Yes	Advanced	DECEASED	32	15.48
28	04-0008-10	Male	62	YES	NO	NO	Tongue	Yes	Advanced	ALIVE	33	1.65
29	11-0004-07	Female	58	NO	NO	YES	Tongue	No	Advanced	DECEASED	11	18.37
30	11-0010-10	Female	35	NO	NO	NO	Tongue	No	Early	ALIVE	30	22.21
31	01-0005-09-1	Female	71	NO	NO	NO	Non tongue	No	Advanced	ALIVE	24	2.68
32	06-0029-08-1	Female	86	YES	NO	NO	Non tongue	Yes	Advanced	DECEASED	8	0.8
33	01-0004-09-1	Female	75	NO	NO	YES	Non tongue	No	Advanced	ALIVE	14	56.82
34	01-0036-08-1	Female	63	NO	YES	YES	Non tongue	Yes	Advanced	ALIVE	42	237.74
35	01-0002-10-1	Male	72	NO	NO	YES	Non tongue	No	Advanced	LOF	9	19.54
36	06-0005-10-1	Female	63	NO	YES	YES	Non tongue	No	Early	DECEASED	3	37.88
37	06-0027-09-1	Male	62	NO	YES	YES	Non tongue	Yes	Advanced	DECEASED	4	11.73
38	01-0022-07-1	Male	49	YES	NO	NO	Non tongue	No	Advanced	LOF	39	17.11
39	01-0024-08-1	Female	61	NO	NO	YES	Non tongue	Yes	Advanced	ALIVE	33	48.52
40	01-0007-09-1	Female	61	NO	NO	YES	Non tongue	Yes	Advanced	LOF	3	21.68
41	04-0019-05-1	Female	55	NO	NO	YES	Non tongue	No	Early	LOF	52	0.42
42	04-0014-08-1	Male	60	YES	NO	NO	Non tongue	Yes	Advanced	ALIVE	40	3.68
43	11-0002-08	Female	71	NO	NO	YES	Non tongue	No	Early	DECEASED	12	114.19
44	01-0058-07	Female	66	YES	YES	NO	Non tongue	No	Early	ALIVE	35	103.72
45	01-0107-08	Female	65	YES	NO	NO	Non tongue	No	Advanced	DECEASED	15	1.32
46	01-0106-08	Male	20	NO	YES	NO	Non tongue	Yes	Advanced	DECEASED	12	4.51

NO	IRPA	GENDER	AGE	SMOKE	DRINK	CHEW	TUMOR SUBSITE	LNM	TUMOUR STAGE	ALIVE/ DEATH	MONTHS	RQ ISG15
47	01-0022-10	Female	51	NO	NO	NO	Non tongue	Yes	Advanced	DECEASED	9	12.9
48	01-0005-12	Female	49	NO	NO	NO	Non tongue	Yes	Advanced	DECEASED	1	41.34
49	01-0019-10	Female	76	NO	NO	YES	Non tongue	No	Early	ALIVE	24	31.51
50	01-0012-11	Female	57	NO	NO	NO	Non tongue	No	Early	ALIVE	17	89.17
51	06-0032-08	Male	48	YES	NO	YES	Non tongue	Yes	Advanced	DECEASED	8	2.53
52	06-0033-10	Female	72	NO	NO	YES	Non tongue	Yes	Advanced	DECEASED	6	208.16
53	06-0055-10	Female	60	NO	YES	YES	Non tongue	No	Early	ALIVE	25	139.18
54	06-0002-11	Female	75	NO	NO	YES	Non tongue	Yes	Advanced	DECEASED	1	29.99
55	06-0028-11	Female	71	NO	NO	YES	Non tongue	No	Early	ALIVE	18	28.73
56	06-0009-11	Female	41	NO	NO	YES	Non tongue	Yes	Advanced	DECEASED	9	29.25
57	06-0031-11	Female	64	NO	YES	YES	Non tongue	Yes	Advanced	DECEASED	2	40.5
58	04-0022-09	Female	70	NO	NO	NO	Non tongue	No	Advanced	ALIVE	12	1.46
59	04-0040-10	Female	79	NO	NO	YES	Non tongue	No	Early	DECEASED	18	3.79
60	04-0005-09	Female	59	NO	YES	YES	Non tongue	No	Early	ALIVE	33	25.09
61	04-0013-11	Female	65	NO	NO	YES	Non tongue	Yes	Advanced	ALIVE	19	5.43
62	04-0002-10	Male	66	NO	NO	NO	Non tongue	Yes	Advanced	DECEASED	12	1.92
63	11-0013-10	Male	58	YES	YES	NO	Non tongue	No	Advanced	ALIVE	28	16.81
64	11-0015-10	Female	59	YES	NO	NO	Non tongue	No	Advanced	ALIVE	26	3.57

APPENDIX G: DETAIL OF REVERSE TRANSCRIPTION CDNA SYNTHESIS PROTOCOL

cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Total of 2 µg pf total extracted RNA were adjusted into final volume of 50 µl with nuclease free water and mixed with 50 µl 2X reverse transcription (RT) master mix. The reverse transcription master mix was set-up as follows:

Reverse transcription PCR ingredients.

Component	Volume/Reaction (µl)
10 X RT buffer	10
25 X dNTPs mix (100 mM)	4
10 X RT random primers	10
MultiScribeTM RT (50U/µl)	5
Nuclease free water	21
Total per reaction	50

Reverse transcription was performed by setting the thermal cycler conditions as below:

Reverse transcription PCR conditions.

	Step 1	Step 2	Step 3	Step 4
Temperature (º C)	25	37	85	4
Time	10 min	120 min	5 sec	∞

APPENDIX H: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE OSCC PATIENTS FOR MMP13 PROTEIN EXPRESSION IHC STUDY

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOUR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	MMP13 SCORE
1	D119/06	61	Female	Yes	No	No	Buccal	2	no	2	Early stage	Non-cohesive	Well	Dead	15	7
2	D137/04	70	Female	Yes	No	No	Buccal	2	no	3	Late stage	Non-cohesive	Mod	Dead	21	4
3	D142/09	49	Female	Yes	No	No	Tongue	1	no	1	Early stage	Non-cohesive	Mod	Alive	35	6
4	D139/04	60	Female	Yes	No	No	Buccal/Palate	4	yes	4	Late stage	Non-cohesive	Well	Dead	21	7.67
5	D139/05	70	Female	Yes	No	No	Buccal/Gum	4	yes	4	Late stage	Non-cohesive	Mod	Dead	66	4.67
6	D146/08	49	Male	No	Yes	No	Tongue	1	no	1	Early stage	Non-cohesive	Well	Alive	47	3.33
7	D150/08	80	Male	No	Yes	Yes	Tongue	1	no	1	Early stage	Non-cohesive	Mod	Dead	24	2
8	D164/04	72	Male	No	No	Yes	Tongue	2	no	2	Early stage	Non-cohesive	Mod	Dead	19	5
9	D406/07	74	Female	No	No	Yes	Gum	4	yes	4	Late stage	Non-cohesive	Well	Dead	7	5.33
10	D193/08	66	Female	Yes	No	Yes	Buccal	2	no	2	Early stage	Non-cohesive	Well	Alive	47	6.67
11	D212/05	59	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Well	Dead	26	6
12	D75/08	70	Female	No	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Mod	Dead	19	6
13	D250/05	76	Male	No	No	Yes	Tongue	1	no	1	Early stage	Non-cohesive	Mod	Alive	80	4
14	D264/06	63	Female	No	No	No	Tongue	1	no	4	Late stage	Non-cohesive	Mod	Dead	12	5.33
15	D268/08	44	Male	Yes	No	No	Gum	4	yes	4	Late stage	Non-cohesive	Well	Dead	12	4.33
16	D287/07	49	Female	No	No	No	Tongue	3	no	3	Late stage	Non-cohesive	Mod	Alive	57	4.67
17	D335/03	55	Male	No	No	No	Buccal	1	no	1	Early stage	Cohesive	Mod	Dead	38	3.33
18	D373/09	67	Male	No	Yes	Yes	Tongue	2	no	2	Early stage	Non-cohesive	Mod	Alive	53	2.67
19	D379/04	64	Male	Yes	No	Yes	Lip	1	no	1	Early stage	Non-cohesive	Well	Alive	92	5.33
20	D388/08	56	Female	Yes	No	Yes	Buccal	3	no	4	Late stage	Non-cohesive	Mod	Dead	37	4
21	D390/06	58	Female	Yes	No	No	Mandible	1	no	1	Early stage	Cohesive	Well	Alive	65	3.33
22	D393/07	50	Male	No	No	Yes	Tongue	4	yes	4	Late stage	Non-cohesive	Mod	Alive	55	3.33
23	D410/07	60	Female	Yes	No	No	Tongue	2	no	4	Late stage	Non-cohesive	Well	Dead	3	5.33
24	D422/04	41	Male	No	Yes	No	Gum, Buccal	2	no	4	Late stage	Non-cohesive	Mod	Alive	89	7.67

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOUR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	MMP13 SCORE
25	D422/05	28	Female	No	No	No	Tongue	1	no	1	Early stage	Cohesive	Well	Alive	76	2.67
26	D442/06	66	Female	Yes	No	No	Gum	4	yes	4	Late stage	Cohesive	Well	Dead	60	6.67
27	D48/06	59	Female	Yes	No	Yes	Buccal	2	no	4	Late stage	Non-cohesive	Mod	Alive	43	6.67
28	D508/07	41	Female	No	No	No	Tongue	2	no	3	Late stage	Non-cohesive	Mod	Dead	17	7
29	D525/06	61	Female	Yes	No	No	Buccal	2	no	2	Early stage	Non-cohesive	Well	Dead	9	5
30	D67/07	49	Male	No	Yes	Yes	Tongue	1	no	1	Early stage	Cohesive	Well	Alive	15	5
31	D68/05	67	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Well	Dead	10	5
32	D184/07	67	Male	No	No	No	Gum	4	yes	4	Late stage	Non-cohesive	Mod	Dead	4	4
33	D146/04	80	Female	Yes	No	Yes	Gum	4	yes	4	Late stage	Cohesive	Well	Alive	93	3.33
34	D517/04	70	Female	Yes	No	No	Buccal	3	no	3	Late stage	Non-cohesive	Mod	Alive	20	7.67
35	D273/06	49	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Cohesive	Mod	Alive	67	8.33
36	D498/06	58	Female	Yes	No	No	Gum	3	no	4	Late stage	Non-cohesive	Well	Dead	5	6.33
37	D158/10	53	Male	No	Yes	Yes	Tongue	2	no	4	Late stage	Non-cohesive	Mod	Dead	13	4
38	D353/09	62	Male	Yes	No	Yes	Buccal	4	yes	4	Late stage	Non-cohesive	Mod	Dead	10	8
39	D366/06	67	Female	Yes	No	Yes	Buccal	1	no	4	Late stage	Non-cohesive	Well	Alive	65	2.67
40	D226/10	59	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Mod	Dead	21	8
41	D419/08	48	Male	No	Yes	Yes	FOM	4	yes	4	Late stage	Non-cohesive	Well	Dead	6	5.33
42	D553/10	49	Female	Yes	No	No	Buccal	1	no	3	Late stage	Non-cohesive	Mod	Alive	16	2
43	D113/04	66	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Mod	Dead	77	4.67
44	D210/05	36	Male	No	Yes	No	Gum	4	yes	4	Late stage	Non-cohesive	Mod	Dead	8	3
45	D304/09	60	Female	Yes	No	No	Tongue	2	no	4	Late stage	Non-cohesive	Mod	Dead	6	4.67
46	D48/05	50	Male	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Well	Alive	75	6.67
47	D474/04	50	Female	Yes	No	No	Buccal	2	no	3	Late stage	Non-cohesive	Mod	Dead	44	7.67
48	D601/08	56	Male	No	Yes	Yes	Buccal	2	no	2	Early stage	Non-cohesive	Well	Alive	38	1.67

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOUR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	MMP13 SCORE
49	D371/07	60	Female	Yes	No	Yes	Buccal	4	yes	4	Late stage	Non-cohesive	Well	Dead	11	8
50	D507/07	52	Female	Yes	No	No	Tongue	4	yes	4	Late stage	Non-cohesive	Mod	Alive	65	7.33
51	D476/06	38	Male	No	Yes	Yes	Tongue	4	yes	4	Late stage	Non-cohesive	Mod	Dead	21	4
52	D289/07	73	Female	No	No	No	Tongue	1	no	1	Early stage	Non-cohesive	Poor	Alive	12	4
53	D461/10	36	Female	No	No	No	Tongue	1	no	1	Early stage	Non-cohesive	Mod	Alive	16	2.67
54	D406/10	74	Female	Yes	No	No	Gum	3	no	4	Late stage	Non-cohesive	Mod	Dead	13	4
55	D412/09	42	Male	No	Yes	No	Tongue	2	no	2	Early stage	Non-cohesive	Mod	Alive	22	6
56	D230/09	73	Female	Yes	No	Yes	Tongue	1	no	1	Early stage	Non-cohesive	Well	Dead	5	4
57	D416/09	74	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	Well	Dead	6	3.33
58	01-0022-10	51	Female	No	No	No	Gum	4	yes	4	Late stage	Non-cohesive	Well	Dead	9	9
59	01-0001-06	71	Female	Yes	No	No		4	yes	4	Late stage	Non-cohesive	Well	Dead	2	4
60	01-0019-10	76	Female	Yes	No	No	gum	1	no	1	Early stage	Non-cohesive	mod	Alive	24	8
61	01-0024-10	29	Male	No	Yes	No	Tongue	1	no	1	Early stage	.	poor	Alive	14	8
62	04-0005-09	59	Female	Yes	No	Yes	buccal	2	no	2	Early stage	.	Well	Alive	33	12
63	04-0013-11	65	Female	Yes	No	No		3	no	4	Late stage	.	Well	Alive	19	6
64	04-0019-05	55	Female	Yes	No	No	Buccal	2	no	2	Early stage	.	Mod	Alive	52	1
65	04-0040-10	79	Female	Yes	No	No		2	no	2	Early stage	.	we	Dead	18	3
66	06-0002-11	75	Female	Yes	No	No	Buccal	2	no	4	Late stage	Non-cohesive	mod	Dead	1	12
67	11-0005-07	34	Male	No	Yes	No		3	no	3	Late stage	.	we	Dead	13	12
68	11-0015-10	59	Female	No	Yes	No	buccal	3	no	3	Late stage	.	mod	Alive	26	8
69	04-0026-08	29	Female	No	No	No	Tongue	1	no	3	Late stage	.	Well	Dead	32	4
70	04-0007-08	52	Female	No	No	No		2	no	2	Early stage	.	Well	Alive	48	12
71	06-0007-11	51	Female	No	No	No		1	no	4	Late stage	Non-cohesive	well	Alive	12	8
72	01-0009-11	61	Female	No	No	No		3	no	4	Late stage	.	well	Dead	12	6

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOUR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	MMP13 SCORE
73	01-0025-12	62	Male	No	Yes	Yes		1	no	4	Late stage	Non-cohesive	mod	Alive	7	12
74	01-0002-10	72	Male	Yes	No	No	buccal	4	yes	4	Late stage	.	Well	Alive	9	2
75	01-0003-10	57	Female	Yes	No	No		2	.	2	Early stage	.	WELL	Alive	36	8
76	01-0023-08	80	Male	No	Yes	Yes		1	.	1	Early stage	Non-cohesive	MOD	Dead	24	9
77	01-0024-08	61	Female	Yes	No	No		2	.	4	Late stage	Cohesive	MOD	Alive	57	6
78	01-0106-08	20	Male	No	No	Yes		4	.	4	Late stage	.		Dead	12	4
79	04-0026-08	29	Female	No	No	No		1	.	4	Late stage	.	WELL	Dead	32	6
80	01-0026-06	58	Female	No	Yes	Yes	BUCCAL	1	.	1	Early stage	.	WELL	Alive	77	6
81	01-0043-11	63	Female	Yes	No	No	GUM	3	.	3	Late stage	Non-cohesive	MOD	Alive	23	9
82	11-0008-10	58	Male	No	Yes	No	BU	3	.	4	Late stage	.		Dead	11	4
83	11-0049-11	37	Male	No	No	No		4	.	4	Late stage	.		Alive	15	4
84	D184/07	67	Male	No	No	No	gum	4	yes	4	Late stage	Non-cohesive	Mod	Dead	3	12
85	D265/10	77	Female	No	No	Yes	Tongue	1	no	1	Early stage	Cohesive	Mod	Alive	27	6
86	D375/10	56	Female	Yes	No	No	gum	4	yes	4	Late stage	Cohesive	mod	Dead	10	6.67
87	D382/07	88	Male	Yes	No	No	Cheek	2	no	2	Early stage	Non-cohesive	Poor	Alive	48	3
88	D406/04	60	Female	No	No	No	Buccal	2	no	2	Early stage	Non-cohesive	Mod	Dead	20	4.5
89	D418/08	53	Male	Yes	No	Yes	Tongue	1	no	1	Early stage	Cohesive	Well	Alive	54	3
90	D62/06	72	Female	Yes	No	No	Buccal	1	no	1	Early stage	Cohesive	Well	Alive	59	8
91	D93/07	74	Female	Yes	No	No	Cheek	1	.	1	Early stage	Non-cohesive	WELL	Dead	31	6
92	D106/07	52	Male	No	Yes	Yes	Tongue	4	.	4	Late stage	Non-cohesive	Mod	Dead	1	6
93	D182/07	65	Female	Yes	No	No	TONGUE	1	.	.	Late stage	.	WWEL	Dead	1	7
94	D194/07	77	Female	No	No	No	BM	.	.	.	.	.		Dead	11	7
95	D198/10	38	Male	No	No	No	TONGUE	1	no	1	Early stage	Non-cohesive	MOD	Alive	12	8
96	D356/10	89	Female	Yes	No	Yes	GUM	.	.	.	.	Non-cohesive	MOD	Alive	.	5.33

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOUR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	MMP13 SCORE
97	D419/07	66	Female	No	Yes	Yes	BM	2	no	2	Early stage	Non-cohesive	WELL	Alive	45	2
98	D426/10	49	Female	Yes	No	No	LIP	2	no	2	Early stage	Non-cohesive	MOD	Alive	15	3.33
99	D446/10	72	Female	Yes	No	No	BM	4	yes	4	Late stage	Non-cohesive	MOD	Dead	9	4
100	D493/10	49	Female	Yes	No	No	LIP	2	no	2	Early stage	Cohesive	MOD	Alive	29	4.5
101	D426/07	65	Male	No	Yes	Yes	FOM	1	no	4	Late stage	Non-cohesive	MOD	Dead	38	5
102	D607/10	52	Female	No	No	No	TONUE	4	yes	4	Late stage	Non-cohesive	MOD	Dead	13	3.7
103	D609/10	56	Male	No	Yes	Yes	FOM	1	no	4	Late stage	Non-cohesive	MOD	Alive	18	5.33

APPENDIX I: CLINICO-PATHOLOGICAL PARAMETERS AND SOCIO-DEMOGRAPHIC DATA OF THE OSCC PATIENTS FOR ISG15 PROTEIN EXPRESSION IHC STUDY

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	ISG15 SCORE
1	D119/06	61	Female	Yes	No	No	Buccal	2	no	2	Early stage	Non-cohesive	well	Dead	15	10
2	D137/04	70	Female	Yes	No	No	Buccal	2	no	3	Late stage	Non-cohesive	poor and moderate	Dead	21	9
3	D142/09	49	Female	Yes	No	No	Tongue	1	no	1	Early stage	Non-cohesive	poor and moderate	Alive	35	9
4	D139/04	60	Female	Yes	No	No	Buccal/Palate	4	yes	4	Late stage	Non-cohesive	well	Dead	21	4
5	D139/05	70	Female	Yes	No	No	Buccal/Gum	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	66	6
6	D146/08	49	Male	No	Yes	No	Tongue	1	no	1	Early stage	Non-cohesive	well	Alive	47	9
7	D150/08	80	Male	No	Yes	Yes	Tongue	1	no	1	Early stage	Non-cohesive	poor and moderate	Dead	24	1.5
8	D164/04	72	Male	No	No	Yes	Tongue	2	no	2	Early stage	Non-cohesive	poor and moderate	Dead	19	1
9	D406/07	74	Female	No	No	Yes	Gum	4	yes	4	Late stage	Non-cohesive	well	Dead	7	6.67
10	D193/08	66	Female	Yes	No	Yes	Buccal	2	no	2	Early stage	Non-cohesive	well	Alive	47	1
11	D212/05	59	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	well	Dead	26	7
12	D75/08	70	Female	No	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	19	5.33
13	D250/05	76	Male	No	No	Yes	Tongue	1	no	1	Early stage	Non-cohesive	poor and moderate	Alive	80	3
14	D268/08	44	Male	Yes	No	No	Gum	4	yes	4	Late stage	Non-cohesive	well	Dead	12	5.67
15	D287/07	49	Female	No	No	No	Tongue	3	no	3	Late stage	Non-cohesive	poor and moderate	Alive	57	12
16	D335/03	55	Male	No	No	No	Buccal	1	no	1	Early stage	Cohesive	poor and moderate	Dead	38	3.33
17	D373/09	67	Male	No	Yes	Yes	Tongue	2	no	2	Early stage	Non-cohesive	poor and moderate	Alive	53	3
18	D379/04	64	Male	Yes	No	Yes	Lip	1	no	1	Early stage	Non-cohesive	well	Alive	92	3.33
19	D388/08	56	Female	Yes	No	Yes	Buccal	3	no	4	Late stage	Non-cohesive	poor and moderate	Dead	37	1
20	D390/06	58	Female	Yes	No	No	Mandible	1	no	1	Early stage	Cohesive	well	Alive	65	1
21	D393/07	50	Male	No	No	Yes	Tongue	4	yes	4	Late stage	Non-cohesive	poor and moderate	Alive	55	3.67
22	D410/07	60	Female	Yes	No	No	Tongue	2	no	4	Late stage	Non-cohesive	well	Dead	3	4.67
23	D422/04	41	Male	No	Yes	No	Gum, Buccal	2	no	4	Late stage	Non-cohesive	poor and moderate	Alive	89	4.5
24	D422/05	28	Female	No	No	No	Tongue	1	no	1	Early stage	Cohesive	well	Alive	76	3.33

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	ISG15 SCORE
25	D442/06	66	Female	Yes	No	No	Gum	4	yes	4	Late stage	Cohesive	well	Dead	60	3.33
26	D48/06	59	Female	Yes	No	Yes	Buccal	2	no	4	Late stage	Non-cohesive	poor and moderate	Alive	43	7.33
27	D508/07	41	Female	No	No	No	Tongue	2	no	3	Late stage	Non-cohesive	poor and moderate	Dead	17	9
28	D525/06	61	Female	Yes	No	No	Buccal	2	no	2	Early stage	Non-cohesive	well	Dead	9	11
29	D67/07	49	Male	No	Yes	Yes	Tongue	1	no	1	Early stage	Cohesive	well	Alive	15	3.67
30	D68/05	67	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	well	Dead	10	2
31	D184/07	67	Male	No	No	No	Gum	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	4	2.33
32	D146/04	80	Female	Yes	No	Yes	Gum	4	yes	4	Late stage	Cohesive	well	Alive	93	1.5
33	D517/04	70	Female	Yes	No	No	Buccal	3	no	3	Late stage	Non-cohesive	poor and moderate	Alive	88	3
34	D273/06	49	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Cohesive	poor and moderate	Alive	67	5
35	D498/06	58	Female	Yes	No	No	Gum	3	no	4	Late stage	Non-cohesive	well	Dead	5	3.33
36	D158/10	53	Male	No	Yes	Yes	Tongue	2	no	4	Late stage	Non-cohesive	poor and moderate	Dead	13	5.67
37	D353/09	62	Male	Yes	No	Yes	Buccal	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	10	4
38	D366/06	67	Female	Yes	No	Yes	Buccal	1	no	4	Late stage	Non-cohesive	well	Alive	65	3
39	D226/10	59	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	21	5
40	D419/08	48	Male	No	Yes	Yes	FOM	4	yes	4	Late stage	Non-cohesive	well	Dead	6	1.33
41	D553/10	49	Female	Yes	No	No	Buccal	1	no	3	Late stage	Non-cohesive	poor and moderate	Alive	16	3.33
42	D113/04	66	Female	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	77	4
43	D210/05	36	Male	No	Yes	No	Gum	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	8	1
44	D304/09	60	Female	Yes	No	No	Tongue	2	no	4	Late stage	Non-cohesive	poor and moderate	Dead	6	4
45	D48/05	50	Male	Yes	No	No	Buccal	4	yes	4	Late stage	Non-cohesive	well	Alive	75	1
46	D474/04	50	Female	Yes	No	No	Buccal	2	no	3	Late stage	Non-cohesive	poor and moderate	Dead	44	7
47	D601/08	56	Male	No	Yes	Yes	Buccal	2	no	2	Early stage	Non-cohesive	well	Alive	38	2
48	D50707	52	Female	Yes	No	No	Tongue	4	yes	4	Late stage	Non-cohesive	poor and moderate	Alive	65	9

NO	BLOCK	AGE	GENDER	SMOKE	DRINK	CHEW	TUMOR SUBSITE	TUMOR SIZES	LNM	TUMOR STAGE	EARLY/ ADVANCED	POI	DIFFERENTIATION	ALIVE/ DEAD	MONTHS	ISG15 SCORE
49	D476/06	38	Male	No	Yes	Yes	Tongue	4	yes	4	Late stage	Non-cohesive	poor and moderate	Dead	21	4
50	D289/07	73	Female	No	No	No	Tongue	1	no	1	Early stage	Non-cohesive	poor and moderate	Alive	12	2
51	D461/10	36	Female	No	No	No	Tongue	1	no	1	Early stage	Non-cohesive	poor and moderate	Alive	16	3
52	D406/10	74	Female	Yes	No	No	Gum	3	no	4	Late stage	Non-cohesive	poor and moderate	Dead	13	6
53	D412/09	42	Male	No	Yes	No	Tongue	2	no	2	Early stage	Non-cohesive	poor and moderate	Alive	22	3

APPENDIX J: DEMOGRAPHICS AND CLINICO-PATHOLOGICAL CHARACTERISTICS OF PATIENTS FROM WHICH THE CELL LINES USED IN THIS STUDY WERE DERIVED.

Cell lines	Age	Gender	Broader's Differentiation	TNM[a]			Stage	Tumor subsite	Risk habits
ORL-48T	79	Female	Well	4	2	0	IV	Gum	None
ORL-188T	56	Male	Moderate	2	2	X	IV	Tongue	Smoking
ORL-204T	76	Male	Moderate	4	1	X	IV	Buccal Mucosa	Betel quid chewing, Smoking, Alcohol drinking

[a]Largest tumour dimension and node status determined by histopathology examination.

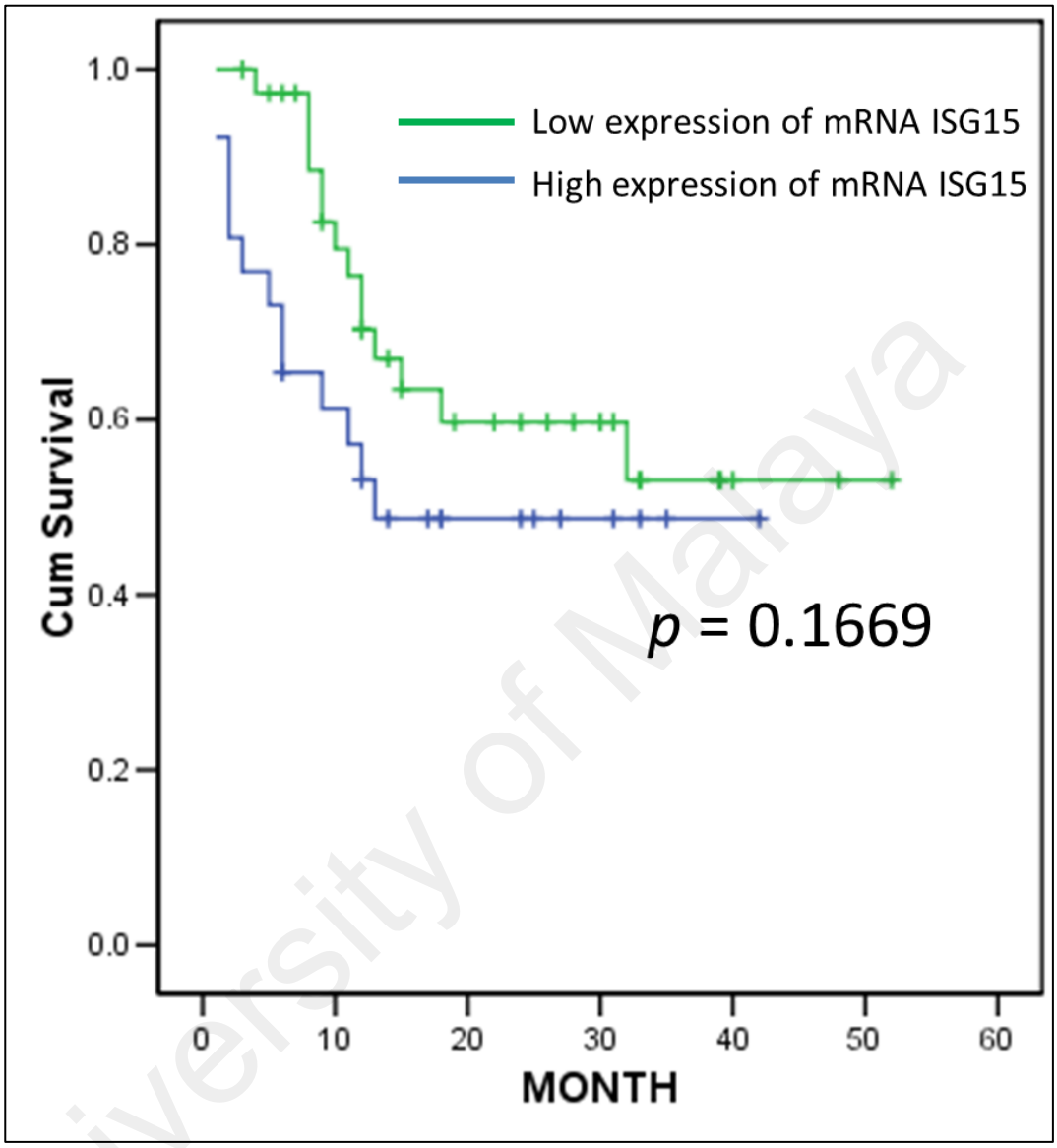
APPENDIX K: ASSOCIATION OF ISG15 MRNA EXPRESSION WITH SOCIO-DEMOGRAPHIC AND CLINICO-PATHOLOGICAL PARAMETERS

Variables	**Category**	**No. of samples (%)**	**Low expression of ISG15**	**High expression of ISG15**	
			No. of samples (%)	**No. of samples (%)**	**p value**
		64	38	26	
Gender	Male	23 (35.9)	18 (47.4)	5 (19.2)	**0.021**
	Female	41 (64.1)	20 (52.6)	21 (80.8)	
Age (years)	< 45	12 (18.8)	10 (26.3)	2 (7.7)	0.057
	≥ 45	52 (81.2)	28 (73.7)	24 (92.3)	
Smoking	No	42 (65.6)	21 (55.3)	21 (80.8)	**0.035**
	Yes	22 (34.4)	17 (44.7)	5 (19.2)	
Drinking	No	48 (75.0)	29 (76.3)	19 (73.1)	0.769
	Yes	16 (25.0)	9 (23.7)	7 (26.9)	
Betel quid chewing	No	36 (56.3)	27 (71.1)	9 (34.6)	**0.004**
	Yes	28 (43.8)	11 (28.9)	17 (65.4)	
Tumour site	Tongue	30 (46.9)	19 (50.0)	11 (42.3)	0.545
	Non-tongue*	34 (53.1)	19 (50.0)	15 (57.7)	
Lymph node status	Negative	31 (48.4)	21 (55.3)	10 (38.5)	0.187
	Positive	33 (51.6)	17 (44.7)	16 (61.5)	
pTNM Staging	Early stage	22 (34.4)	13 (24.2)	9 (34.6)	0.973
	Advanced stage	42 (65.6)	25 (65.8)	17 (65.4)	

*****Buccal mucosa, gingiva, lip, floor of mouth, palate

Significant p - value were highlighted in bold.

APPENDIX L: ASSOCIATION OF ISG15 MRNA EXPRESSION WITH SURVIVAL IN OSCC PATIENTS



Overall survival curves were analyzed according to ISG15 mRNA expression using Kaplan-Meier estimate with log- rank test.