COMPARISON OF LOSS OF HETEROZYGOSITY PATTERNS IN PRIMARY ORAL PREMALIGNANCY AND PREMALIGNANCY ADJACENT TO ORAL CANCER

By

Ming Yeung (Cecil) Chau
B.Sc. (Hon.), University of British Columbia

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the School
of
Kinesiology

© Ming Yeung Chau 2003

SIMON FRASER UNIVERSITY

August 2002

APPROVAL

NAME: Ming Yeung Chau

DEGREE: Master of Science

TITLE OF THESIS: Comparison of Loss of Heterozygosity Patterns in Primary Oral Premalignancy and Premalignancy Adjacent to Oral Cancer

EXAMINING COMMITTEE:

           Chair: **Dr. Christine MacKenzie**
                              Professor

Dr. Miriam Rosin
Senior Supervisor
Professor, School of Kinesiology
Simon Fraser University

Dr. Lewei Zhang
Supervisor
Associate Professor, Oral Biological and Medical Sciences
University of British Columbia

Dr. Wade Parkhouse
Supervisor
Professor, School of Kinesiology
Simon Fraser University

Dr. Michael Nimmo
External Examiner
Clinical Assistant Professor, Pathology & Laboratory Medicine
University of British Columbia

Date Approved: 19th August 2003

PARTIAL COPYRIGHT LICENCE
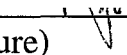
Title of Thesis/Project/Extended Essay

Comparison of Loss of Heterozygosity Patterns in
Primary Oral Premalignancy and Premalignancy
Adjacent to Oral Cancer

Author:

_____
(signature)

_____
Ming Yeung Chau
(name)

_____
28th Aug, 2003
(date)

Abstract

Head and neck squamous cell carcinoma (SCC) is one of the most common human cancers, accounting for about 6% of all cancers in the western world and up to 40% in other parts of the world. SCC is believed to progress through sequential stages of premalignant lesions (OPLs) from hyperplasia to dysplasia (mild, moderate and severe) to carcinoma *in situ* (CIS) before finally becoming invasive SCC. The prognosis of invasive SCC is poor, with a 5-year survival rate of less than 50%. This is primarily due to a high rate of local-regional tumor recurrence in these patients (about 30 to 40%) as well as the development of second primary tumors (17% average). Apparently, the current regimen of surgical removal of at least 1 cm of normal looking oral mucosa beyond the clinical tumor is adequate for some patients but inadequate for others. At present, pathological finding of SCC or CIS or severe dysplasia at the resection margins will invoke aggressive treatment, but there is no guideline for treatment when low-grade dysplasia (mild/moderate) has involved the resection margins. Primary low-grade dysplasia has a low cancer risk; however, it is not clear whether a similar degree of dysplasia found adjacent to a tumor sample has a higher cancer risk.

A recent study from this laboratory has shown that microsatellite analysis for the loss of heterozygosity (LOH) could identify high-risk OPLs. OPLs that later progressed into cancer are characterized by a high frequency of multiple LOH and LOH at 3p &/or 9p plus loss at any of 4q, 8p, 11q, 13q or 17p (RR = 33.4) as compared to OPLs that did not progress into cancer (Rosin *et al.* Clin. Cancer Res. 6:357-362, 2000).

The objective of the study was to compare the cancer risk of 75 primary OPLs (30 hyperplasia and 45 low-grade dysplasia) with 53 OPLs with similar histology but taken adjacent to oral SCC (15 adjacent hyperplasia and 38 low-grade dysplasia) by microsatellite analysis for LOH using the same primers as in our previous study: 3p14.2 (*D3S1234, D3S1228, D3S1300*); 4q26 (*FABP2*); 4q31.1 (*D4S243*); 8p21.3 (*D8S261*); 8p23.3 (*D8S262*); 8p23.3 (*D8S264*); 9p21 (*IFNA, D9S171, D9S1748, D9S1751*); 11q13.3 (*INT2*); 11q22.3 (*D11S1778*); 13q12.3-13 (*D13S170*); 13q14.3 (*D13S133*); 17p11.2 (*CHRNB1*) and 17p13.1 (*tp53* and *D17S786*).

The results showed there was no difference in LOH pattern between primary hyperplasia and hyperplasia taken adjacent to oral SCC. However, compared to primary low-grade dysplasia, low-grade dysplasia taken adjacent to SCC showed increased LOH, particularly multiple LOH: 55% vs. 22% for > 1 LOH, $P = 0.0029$; and 50% vs. 11% for > 2 LOH, $P = 0.0002$. Similarly the high-risk LOH patterns were observed more frequently in low-grade dysplasias adjacent to cancers as compared to the primary lesions: 65% vs. 42% for LOH at 3p and/or 9p, $P = 0.0483$; and 57% vs. 18% for LOH at 3p and/or 9p plus other arms, $P = 0.0004$). Multiple losses and LOH pattern of 3p and/or 9p plus other arms are associated with marked by increased cancer risk. The LOH frequencies on individual arms also differed. Low-grade dysplasia adjacent to cancers had a significant increase in 4q and 11q losses. Our previous studies showed that LOH at 4q and 11q are rare in primary low-grade dysplasia but significantly increased in high-risk lesions such as verrucous hyperplasia and severe dysplasia, or low-grade lesions that

later progressed into cancer (Poh *et al.*, 2000 and Rosin *et al.*, 2000). All of these suggest that low-grade dysplasia adjacent to cancer should not be regarded in general as low-risk lesions as morphologically similar primary lesions.

While dysplasias taken from adjacent to cancer have an overall higher molecular risk as compared to those morphologically similar primary lesions, the results also showed that about half or a little less than half of the low-grade dysplasias adjacent to cancer were molecularly low risk judged by multiple losses (45% had not demonstrated > 1 arm loss and 50% had not demonstrated > 2 arm losses) or by LOH at 3p & 9p plus other chromosome arms (43% did not have such loss). These results again illustrate the value of molecular markers in triage lesions of different risks that allow aggressive treatment of those low-grade dysplasias with high molecular risk to prevent tumor recurrence on one hand but avoid unnecessary aggressive or mutilating surgery on the other.

Acknowledgments

I would like to thank everyone in my lab and my family for supporting me in my study. I also want to give special thanks to Miriam, Lewei and Wan for their teaching and advices. Their passion on science research will motivate me in my career and inspire me for the rest of my life.

Table of contents

List of figures

List of tables

1 Introduction

1.1 Oral Cancer and poor prognosis

Head and neck cancers account for approximately 6% of all cancers in the western world. The incidence is much higher in the Far East, and India in particular, with up to 40% of malignancies occurring in the head and neck region. Despite the recent refinements and improvements in surgery, radiotherapy, and chemotherapy, the prognosis of oral cancer has not significantly improved during the past two decades: the 5-year-survival rate is still less than 50% and is one of the lowest among the major types of cancers (Crowe *et al.*, 2002; Raybaud-Diogene *et al* 1996; Partridge *et al.*, 1998; Tabor *et al* 2001). This poor prognosis is largely due to a high-rate of local regional recurrence as well as development of second primary tumors (SPT) (Khuri *et al.*, 1997; Califano *et al.*, 1996; Lippman and Hong, 1989; Tabor *et al.*, 2001; Van Houten *et al.*, 2002; Vikram *et al.*, 1994). Up to 30% - 40% of oral SCC will recur, the majority within 2 years of treatment (about 70% in the first year and 20% in the second) (BC Cancer Registry). The understanding of the mechanisms of local recurrence is critical for the improvement of the dismal prognosis. The study of premalignant lesions is thus not only important in the intervention and prevention of primary oral SCC but also is one of the keys to understand recurrence and SPT, and such understanding should enable us to manage the high-risk premalignant lesions before they become recurrent or SPT and hence improve the poor prognosis of oral SCC.

1.2   *Etiology of oral cancer*

A combination of environmental and genetic risk factors are involved in the pathogenesis of SCC of the oral cavity and that it involves. The main environmental risk factors of oral cancer are tobacco usage and/or alcohol consumption.

Tobacco usage is the most important etiological factor for HNSCC (Andre *et al* 1995; Bundgaard *et al* 1994; Blot *et al*, 1988; Paterson *et al* 1996). All forms of tobacco - cigarettes, pipes, cigars, and snuff and chewing tobacco - have been implicated in the development of oral cancers (DHEW, 1973; Spitz and Newell, 1987; Winn *et al*, 1981). For example, the incidence of HNSCC is as high as 40% in the Far East and India (Saranath *et al,* 1993), where usage of both smokeless tobacco and smoking is a common habit frequently starting at a young age. Chewing of bedi (tobacco rolled in a temburni leaf) and reverse smoking are also prevalent in these regions.

It is well known that tobacco contains various carcinogens such as aromatic hydrocarbons and nitrosamines. They can form DNA addicts and disturb base-pairing during DNA replication. Chemical analysis reveals that smoke from a single cigarette is composed of over 4,000 different constituents, including some components that are pharmacologically active, toxic, mutagenic, or carcinogenic (DHEW, 1979). For example, polynuclear aromatic hydrocarbons (PAHs) in tobacco smoke have been implicated extensively in oral carcinogenesis, and 4- (methylnitrosamino)-1-(3-pyridyl)-

1-butanone (NNK) and N'- nitrosonornicotine (NNN), which are commonly found in both smokeless tobacco and tobacco smoke and were carcinogenic in rats, mice and Syrian golden hamsters, likely play a major etiological role in oral cancer as well (Brunneman *et al*, 1982; Hoffman *et al*, 1995).

One important phenomenon for exposure to the myriads of carcinogens in tobacco is the 'field' effect. For example, when a person smokes a cigarette, the whole field of oral cavity together with many other organs and tissues (e.g. larynx, pharynx, trachea and lung) are exposed to the carcinogens. Therefore, the whole field that has been exposed to carcinogens will have an increased cancer risk.

The risk associated with alcohol consumption is not as well understood as tobacco. It is not clear whether alcohol by itself is carcinogenic, since heavy drinkers usually tend to smoke as well. Hence, it is difficult to separate the effects of alcohol on carcinogenesis. Some believe that alcohol only acts as a co-carcinogen. The possible mechanisms proposed for the co-carcinogenic effects of alcohol include the dehydrating and irritating effect it has on mucosa, or the theory that it functions potentially as a solvent for carcinogens, making it easier for carcinogen to be absorbed. The co-carcinogenic effects may also result from liver damage by alcohol, which causes reduced clearance of carcinogens. Hsu *et al* (1991) also suggested that ethanol may temporarily inhibit DNA repair. There is no doubt, however, that combined usage of tobacco products and alcohol markedly increases the risk of cancer development.

Beside tobacco and alcohol, other factors such as genetic predisposition and human papilloma virus (HPV) are also related to carcinogenesis of the oral cavity.

The area of genetic predisposition to cancer is becoming more important. Many studies have shown susceptibility could play a major role in determining cancer risk, and markers of susceptibility can be used in association with clinical and histological markers (and the molecular markers discussed in this thesis) to predict cancer risk. However, it is important to note that susceptibility is a complex issue that at present is very poorly understood. Current research suggests that it involves the interactions of multiple genes that produce proteins acting in concert to perform critical events. For example, several polymorphisms have been suggested for genes that code for proteins that are involved in the metabolism of carcinogens. Some of these proteins code for Phase I enzymes and these in general act to activate procarcinogens to active forms that can damage DNA. Such enzymes are normally counterbalanced by the activity of Phase II enzymes (e.g. glutathione, glucuronide, etc.) that act to conjugate various chemical groups to the activated carcinogens to detoxify them, thus preventing DNA damage. The balance between metabolic activation and inactivation, as well as the actual level of carcinogen exposure, are all important aspects involved in determining an individual's risk of developing cancer (Bell *et al*, 1993; Butler *et al*, 1989; Feigelson *et al*, 1996; Frederickson *et al*, 1994). Other areas in which genetic factors may impact on susceptibility include variations in genes that affect proteins that control DNA repair competence (Hsu *et al*, 1983; Schantz and Hsu, 1989; Spitz and Bondy, 1993) or the immune system. Such possibilities are only now being explored.

1.3   *Histology of Oral Mucosa*

The oral cavity is lined with mucosa, which is composed of the overlying stratified squamous epithelium and the underlying lamina propria, commonly known as connective tissue. The underlying connective tissue holds blood and lymphatic vessels as well as nerves and muscle fibers.

The physical barrier that separates the overlying epithelium from underlying connective tissue is called the basement membrane. It is composed of the extracellular matrix, which gives a mechanical support for epithelial cells. This membrane consists of two layers: basal lamina and lamina reticularis. The former is produced by the epithelium, and the latter is produced by connective tissue (Fine, 1991).

The overlying stratified squamous epithelium is composed of three cell types; basal cells, prickle cells, and cornified cells. The cuboidal-shaped basal cells form a very thin layer (usually single-cell layer) that exists between epithelium and connective tissue. The cells within this basal cell layer are the ones that have the capacity to divide and give rise to more new basal cells or differentiate into prickle cells (located in the upper part of the epithelium). As the cells mature, they migrate toward the surface, changing their shapes into more elongated and flattened forms. Once reaching the surface, they will eventually be desquamated.

Like many other parts of the body with mucosa lining, such as the esophagus and cervix, the majority of the oral cavity lining is not keratinized. However, some oral epithelium regions susceptible to mechanical forces such as gingiva and hard plate are covered with keratin and are referred to as masticatory mucosa (Wertz et al., 1993). This mucosa serves as a very effective mechanical and permeability barrier. The dorsum of the tongue is covered with a specialized keratinized epithelium and the mucosa is attached tightly to the underlying tongue muscle (Wertz et al., 1993). The rest of the oral mucosa, such as the buccal mucosa and the floor of mouth, is lined with non-keratinized stratified squamous epithelium and the mucosae are more flexible, thus accommodating the actions of chewing and speaking (Wertz et al., 1993). Over 90% of oral malignancies are squamous cell carcinoma (SCC), arising from this stratified squamous epithelial tissue.

1.4   *Histological Progression Model for Oral Cancer*

Oral SCC is believed to be a result of a long time multistage carcinogenesis process. This multistage process involves progression from normal to premalignant lesions and finally invasive SCC. A premalignant or precancerous lesion has been defined by the World Health Organization (WHO, 1978) as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart. In the oral cavity, premalignant lesions present clinically mostly as leukoplakias, and sometimes as erythroplakia (WHO 1978). Leukoplakia is only a clinical term and means a "white patch" and occurs on mucous membranes of the oral cavity as well as other

organs, such as the mucosa of the oropharynx, larynx, esophagus, and genital tract. Not necessarily white, leukoplakias may also appear yellow to light brown, especially in smokers. The World Health Organization (WHO, 1978) defines leukoplakia in the oral cavity as a white patch or plaque of oral mucosa, which cannot be characterized clinically or pathologically as any other diagnosable disease and cannot be removed by rubbing.

When a biopsy is taken, a leukoplakia will show microscopically hyperkeratosis and/or epithelial hyperplasia (acanthosis) with or without epithelial dysplasia. The World Health Organization has established the following criteria for histological diagnosis of oral dysplasia (1978):

1. Loss of basal cell polarity

2. More than 1 layer of basaloid cells

3. Increased nuclear to cytoplasmic ratio

4. Drop-shaped rete ridges

5. Irregular stratification

6. Increased and abnormal mitoses

7. Mitotic figures in the superficial half of the epithelium

8. Cellular pleomorphism (variation in shape and size)

9. Nuclear hyperchromatism (dark staining nuclei)

10. Enlarged nucleoli

11. Reduction of cellular cohesion

12. Keratinization of single cells or cell groups in the spinous cell layer

Pathologically, dysplastic lesions are further divided into mild, moderate, and severe forms depending upon how much of the epithelial tissue are dysplastic. In mild dysplasia, the dysplastic cells are confined to the basal layer and the cells exhibit the smallest degree of above described changes. With moderate and severe dysplasia, the epithelial layers involved and the severity of the cellular changes are progressively increased. In carcinoma *in situ*, the dysplastic cells occupy the entire thickness of the epithelium (bottom to top changes) although the basement membrane is still intact. Invasion of the dysplastic cells through the basement membrane into the underlying stroma and/or the dissemination of these cells to other sites through lymphoid and circulatory systems are events associated with development of invasive SCC.

The presence or absence of dysplasia, and the degree of dysplasia, is believed to have a huge impact on the malignant risk of the premalignant lesions. All studies to date have shown that leukoplakia with dysplasia has a higher malignant risk than leukoplakia without dysplasia. A large clinical study by Silverman *et al* (1984) found that during a mean onset of 7.2 years after presentation, more than 36% of leukoplakia lesions with microscopic epithelial dysplastic features eventually underwent malignant transformation whereas leukoplakia without dysplasias only demonstrated a malignancy rate of 15%. The risk of dysplasia and degree of dysplasia is further demonstrated by studies from the uterine cervix and other systems and organs including skin and respiratory system. As a result, currently the gold standard for judging the malignant potential of premalignant

lesions in these organs and systems, including the oral cavity, is the presence and degree of dysplasia.

Using these criteria, a histological progression model has been established for the oral cavity (Fig. 1). Premalignant lesions are classified histologically into categories with progressively increased risk of becoming invasive SCC: epithelial hyperplasia (without dysplasia), mild, moderate and severe dysplasias, and carcinoma *in situ* (CIS).

Figure 1. Histological progression model of oral premalignant and malignant lesions
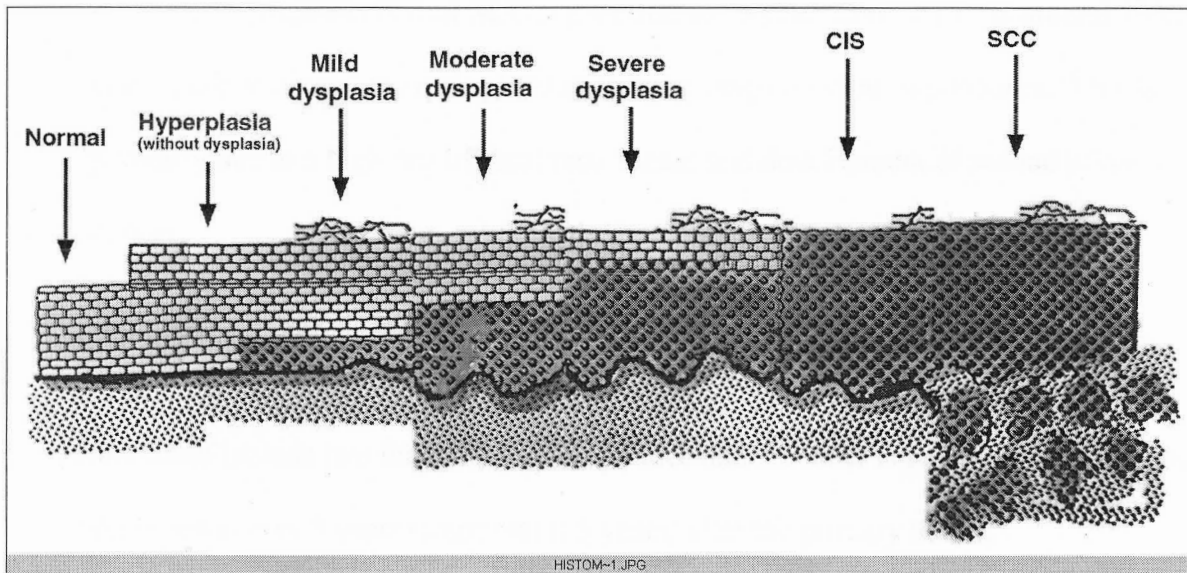
Other factors also affect the malignant potential of oral premalignant lesions. These include location and duration of the lesion, gender of the patient, appearance of the lesion (homogenous versus non-homogenous), and presence of *Candida albicans* (Waal *et al.*, 1997). Most studies on the malignant transformation of oral premalignant lesions

have been done on leukoplakias (clinical presentation of oral premalignant lesions), frequently without knowledge of dysplasia for all the study cases. The reported malignant risk for leukoplakia varies from study to study, ranging from as low as 0.13% to as high as 50% depending upon the patient population and follow-up time (Lumerman *et al.*, 1995; Papadimitrakopoulou and Hong, 1997; Schepman *et al.*, 1998; Silverman *et al.*, 1984).

1.5   *Tumor recurrence and second oral malignancy (SOM)*

The prognosis of oral SCC is poor and the 5-year survival rate is around 50%, one of the poorest among the major human cancers, despite technical advances. This is primarily due to a high rate of local recurrences and development of second primary tumors.

If there is a tumor occurring from the previous tumor site (the index tumor site), this could include two things: one is recurrence and the other is second primary tumor if the lesion occurs 3 years (some call it 5 years) after the primary tumor.

When new tumors appear at the previous oral SCC sites (the index tumor sites), they mostly represent tumor recurrences that develop from the outgrowth of residual tumor cells or high-risk premalignant cells left behind. The latter has also been called second field tumors (SFT), a term proposed by Braakhuis and his colleagues (2002) for those derived from the same genetically altered mucosal field as the primary tumors. In

addition, these tumors could also represent SPTs and local metastasis. To differentiate recurrences from SPTs, most investigators currently use the criteria of that Warren and Gates that were published in 1932. A recurrent tumor is defined to be at the site of the index tumor or a contiguous site of the index tumor and occur within a certain time interval of the index tumor. A second tumor is called a SPT when the second malignancy is histologically different from the index tumor or topographically or chronologically distinct from it, and the probability of one being a metastasis of the other can be excluded.

The histological evaluation for assessment of malignancies and similarity between the index and the second tumor is easy and well accepted. Histological assessment could easily identify second primary tumors if the types of malignancies are different. For example, if the index tumor is a squamous cell carcinoma and the second tumor from the same site is an adenocarcinoma, then the second tumor could not be a recurrence of the index tumor but is a second primary tumor. On the other hand, if the second tumor is also a squamous cell carcinoma of similar or close grade, then histologically the possibility of a recurrence cannot be ruled out.

The criterion of topographical distinction for differentiation between recurrences and second primary tumors is under debate. There is no common agreement on what distance should lie between the index tumor and the second tumor. Some say 1.5 cm (Scholes *et al.*, 1998), others take at least 2 cm (Hong *et al.*, 1990; Shin *et al.*, 1996, van de Tol *et al.*, 1999), while more recent studies suggest at least 3 cm (Tabor *et al.*, 2002).

The criterion of chronological distinction for differentiation between recurrences and second primary tumors is also under debate. Since the vast majority of SCC recurs within 5 years of treatment, tumors developed from the same site beyond the 5-year span may be regarded as a new tumor. There is, however, no hard evidence available to support this presumption (Funk *et al.*, 2002). More recent studies have shown that the chronological distinction time should be 3 years (Leong *et al.*, 1998; Shin *et al.*, 1996). Still others do not believe in the chronological distinction and one group proposed that any subsequent SCC at the index tumor site or direct vicinity (<2 cm) of the indexed primary tumor, regardless of the time from the primary tumor, should be considered as a recurrence (van de Tol *et al.*, 1999).

The difficulty in differentiation between recurrences and second primary tumors has been demonstrated by recent molecular evidence that tumors defined by the above described traditional criterion as second primary tumors could in reality be tumor recurrence (see discussion bellow). Furthermore, the differentiation of local metastasis from recurrence could be even harder. The term "second oral malignancy" (SOM) has been coined for second tumors occurring at the index tumor sites without differentiating between recurrences and SPTs.

1.6   *Development of second oral malignancies*

There are two theories regarding the development of SOMs (recurrences and SPTs): the traditional field cancerization theory and the clonal expansion theory.

1.6.1   Traditional concept of field cancerization

The prevailing theory that supports the usage of topographical distinction as a criterion in the differentiation of recurrent tumor from second primary tumor is the field cancerization theory proposed by Slaughter *et al.* in 1953. According to this theory, the whole field, for example the oral cavity, under the repeated exposures of carcinogens will undergo multiple independent genetic alterations, which could give rise to multiple tumors unrelated to each other. The following points were used as supporting evidence: 1) several distinct lesions within the same resection specimen, 2) margins of normal mucosa around a tumor with another noncontiguous area of dysplasia or contiguous area of dysplasia, 3) several small distinct premalignant lesions could coalesce to form a tumor; 4) the high incidence of second primary tumors found in this patient population.

1.6.2   One clone expansion theory

On the other hand, more recent studies have shown that topographically quite distinct tumors (classified traditionally as second primary tumors) share some or even all genetic changes indicating the two tumors are related (Califano *et al.*, 1999; Partridge *et al.*, 2001). Sidransky *et al.* (1992) studied patients with multifocal bladder carcinoma and found that all tumors seemed to have arisen from one clonal population. Bedi *et al.* (1996) later found out the same clonal expansion also happened in HNSCC. Additional evidence in a small group of head and neck SCC patients suggested that at least a proportion of multiple SCC arise from a single clone. Although the concept that precancerous change extends beyond the macroscopically visible tumor is widely accepted, the extensive expansion of a single clone is only recently scientifically demonstrated.

Califano *et al.* in 1999 examined mucosa surrounding invasive head and neck tumors and found similar genetic mutations occurring in the histologically abnormal mucosa as were present in the tumor. Thus similar events are occurring in surrounding mucosa with histologic abnormalities and the adjacent invasive tumor. At least a portion of these appears to involve a clonal pattern of growth from a single progenitor cell. They also found that a squamous cell carcinoma of the esophagus was similar genetically to an oropharyngeal squamous cell carcinoma in one patient (Califano *et al.*, 1999).

Based on these data, Sidransky *et al* has proposed an alterative hypothesis, the clonal expansion theory (Califano *et al.*, 1999, 2000; Mao *et al.*, 1994; Sidransky *et al.*, 1992b). This theory states that in some patients, instead of multiple independent genetic

events, there are widespread clonal migrations of one genetic event through the whole aerodigestive tract. This can be through migration of tumor cell, for example, by saliva (micrometastases), and by intraepithelial migration of the progeny of the initially transformed cells. With this theory, topographically distinct tumors could either be 'identical' (hence tumor recurrence) or partially related (that is, the new tumor is derived from similar premalignant field as the first tumor and hence share the early genetic events).

In summary, both the traditional field cancerization theory and the new clonal expansion theory indicate that the mucosa surrounding the oral squamous cell tumor could contain genetic/morphological abnormalities.

In this study, dysplasias adjacent to SCC were examined to determine the risk for the adjacent dysplasia to progress into cancer in the future. In addition, by looking into the different genetic alterations between SCC and their adjacent dysplasia, we may have a better understanding of field cancerization and clonal expansion.

1.7   *Treatment of oral SCC: Should low-grade dysplasia be left?*

Treatment of primary oral tumors in British Columbia Canada usually involves surgery, irradiation or both.

The surgical removal of oral SCC aims for at least 1 cm of normal-looking mucosa margin (Al-Rajhi *et al.*, 2000; Juan *et al.*, 2001) although frequently only 5 mm margin is included because of the location of the tumor and impossibility of removal of a large normal-looking mucosa margin (Brown *et al.*, 2002; Tabor *et al.*, 2001). For radiation therapy, the rate of radiation delivery, fraction size, field of radiation, and radiation source varies depending on individual patient status and previous surgical intervention. Patients may undergo radiation therapy for 3-7 weeks with a total dose ranging from 3,000 to >7,000 centigray (cGy), depending on tumor type and location (BC Cancer Agency Dentistry website).

The treatment of a second primary tumor (SPT) is similar to a primary tumor, but the chance of recurrence must be ruled out first. In B.C., recurrence of any tumor is defined as tumor appears within 3 years after the first diagnosis, and the location of the second tumor is within 3 cm of the first one. Recurrence is treated aggressively disregarding the stage and grade of the tumor. Treatment usually involves the combination of surgery and radiotherapy (BC Cancer agency website).

Surgical resection margins are evaluated histologically. Currently, if high-grade dysplasia (e.g. severe dysplasia) has involved the resection margin, aggressive treatment will be employed to eradicate these high-grade dysplastic changes because of the high likelihood of cancer progression and the development of tumor recurrences. However, when low-grade dysplasia has involved the biopsy margin, there is no general agreement on whether further treatment is needed. Therefore, many of these lesions are left

untreated. It is not clear whether these low-grade dysplastic changes have higher cancer risk than those morphologically similar but from patients without a history of cancer. If these low-grade lesions are of high cancer risk, they may contribute to the high recurrence of oral SCC if left untreated. This thesis will try to address this issue by comparing the molecular profiles of low-grade dysplasias from adjacent to oral SCC and those primary low-grade dysplasias. In the following section, genetic changes in oral premalignant and malignant lesions will be reviewed.

1.8   Genetic changes found in oral cancer

It is now well established that clonal evolution of cancer is due to a progressive accumulation of critical genetic alterations. The genes include at least two large groups: oncogenes and tumor suppressor genes (TSGs).

Oncogenes are derived by the mutation of normal cellular genes termed proto-oncogenes. Proto-oncogenes act in a dominant fashion to positively regulate cell growth and differentiation. They include genes for growth factors, growth factor receptors, protein kinases, signal transducers, nuclear phosphoproteins, and transcription factors. Mutation of these proto-oncogenes to oncogenes can alternate the structure and activity in coded proteins. Many oncogenes have been identified in the literature; however, few of them have been reported to express in HNSCC. Some of the oncogenes that have been found altered or expressed at abnormal levels in head and neck cancers are *ras, cyclin-D1, myc, erbB, bcl-1, bcl2, int-2* (Frederick *et al.*, 1999; Namazie *et al.*, 2002; O-

charoenrat *et al.*, 2002; Roh *et al.*, 2000; Squire *et al.*, 2002; Takes *et al.*, 2001; Xu *et al.*, 1998).

In contrast, TSGs function antagonistically with cellular proto-oncogenes to negatively regulate cell growth and differentiation. The functions of TSGs must be lost in order for tumorigenesis to occur. According to Knudson's hypothesis (1985), both copies of a tumor suppressor gene have to be inactivated for its protective function to be lost in a cell. Current literature suggests that this process involves two separate events, the first quite often involving a point mutation in one allele, followed by loss of loci containing the wild type gene in the remaining allele. Some of the TSGs involved in head and neck cancers include *p53, Rb (retinoblastoma), and p16INK4A* (Gallo *et al.*, 1999;; Jares *et al.*, 1999; Liggett *et al.*, 1999; Papadimitrakopoulou *et al.*, 1997; Partridge *et al.*, 1999; Sartor *et al.*, 1999; Van Houten *et al.*, 2002; Weber *et al.*, 2002). Other potential candidates are *FHIT (*fragile histidine triad), *APC* (adenomatous polyposis coli), *doc-1, VHL* (the gene responsible for von Hippel-Lidau syndrome) and *TGFBR2* (the gene coding for transforming growth factor type II receptor) (Mao *et al.*, 1998 and 1996; Mao EJ *et al.*, 1998; Paterson *et al.*, 2001; Pavelic *et al.*, 2001; Todd *et al.*, 1995; Uzawa *et al.*, 1994; Waber *et al.*, 1996).

This meticulous balance between growth inducers (coded by proto-oncogenes) and suppressors (coded by tumor suppressor genes) controls the rate of division in normal cells. These genes are altered through a multistep process in which a cell accumulates many genetic changes, breaking the balance of normal cell growth and leading to the

malignant phenotype. Recent advancement in the techniques of molecular analysis has rapidly revolutionized our ability to look at these genetic alterations. My research will focus on loss of tumor suppressor genes (TSGs).

Functional loss of TSGs is one of the most common genetic alterations during carcinogenesis. Therefore, defining chromosomal regions harboring biologically important suppressor genes may have broad practical implications not only on our comprehension of progression of tumors but also on the clinical management of cancers and premalignant lesions. This thesis has studied regions of chromosome loss that contain presumptive TSGs by employing a polymerase chain-based *microsatellite analysis* for *loss of heterozygosity (LOH)*.

1.9   Loss of heterozygosity (LOH) as a genetic test

1.9.1 What is LOH?

LOH is defined as a loss of genomic material (as small as a few thousand nucleotides to as large as a whole chromosome) in one of a pair of chromosomes. The LOH assay is designed to assess polymorphic chromosomal regions that map close to or within putative or known recessive cancer-related genes. The concept of LOH is consistent with Knudson's two-hit hypothesis, which states that inactivation of one of the two alleles by either a germline or somatic mutation will provide a growth advantage to a

tumor cell because only one more inactivation of the remaining allele is needed. LOH analysis has been employed as a means of identifying critical loci containing TSGs, which subsequently led to the discovery of several important TSGs, including the retinoblastoma (*Rb*) gene and the genes responsible for multiple endocrine neoplasia type 1 (*MEN1*), the nevoid basal cell carcinoma syndrome (*NBCCS*), adenomatous polyposis coli (*APC*), and neurofibromatosis type I and II (*NF1 and NFII*, respectively) (Ah-See *et al.*, 1994 and Fearon *et al.*, 1997).

Two methods have been available for the study of LOH or allelic loss: the more classical approach of restriction frequent length polymorphism (RFLP) analysis, and the newer method of microsatellite analysis. This thesis employed microsatellite analysis for at least two reasons. First, microsatellite repeat markers are highly polymorphic and well-distributed throughout the human genome. They show levels of heterozygosity between 30-80%, significantly above the level observed with the RFLP analysis based on base substitutions at endonuclease recognition sites. Second, this PCR-based approach is much more sensitive than the RFLP analysis and requires only small quantities of DNA (5 nanograms or less per reaction). For these reasons, the microsatellite analysis procedure has become the major tool for the majority of current LOH studies of oral premalignant lesions, which tend to be small lesions compared to invasive SCC.

Microsatellites contain runs of short and tandemly iterated sequences of di, tri, or tetranucleotides, such as GTGTGT… or GTAGTAGTA… or GTACGTACGTA…. These short repetitive DNA sequences are called microsatellites. The number of such

tandem repeats is found to be highly polymorphic in the population, with each individual typically containing a different number of copies (generally 4 to 40) of the repeat at each particular locus. In addition, they are well interspersed throughout the human genome (e.g., estimated every 30-60 KB for CA repeats) and are highly conserved through successive generations (Ah-See *et al.,* 1994). Testing of highly polymorphic microsatellite markers from a specific chromosomal region allows rapid assessment of allelic loss by comparing the alleles in tumor DNA to normal DNA. Therefore microsatellites are a good way to research the TSGs either close to or within these chromosome spots. Loss of heterozygosity suggests that a putative tumor suppressor gene nearby is also lost.

Figure 2. Schematic view of loss of heterozygosity

Normal cells   Dysplastic cells
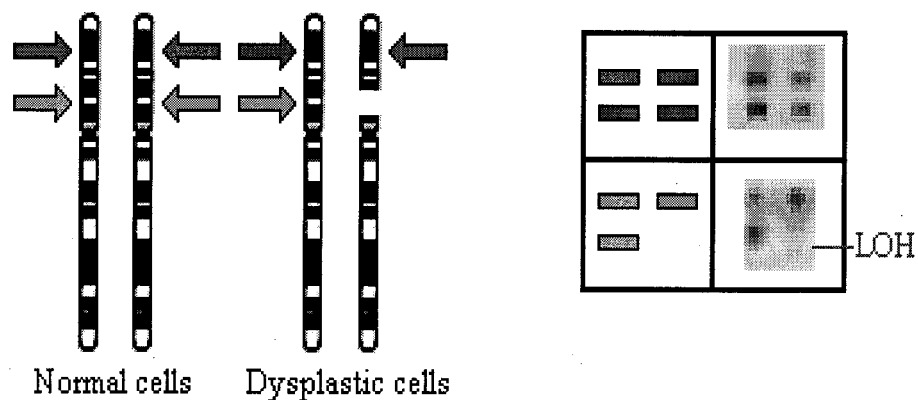
1.9.2 LOH in oral cancer

Recent studies including those from this lab have shown that the loss of specific regions of chromosomes that contain tumor suppressor genes is a common event in oral SCCs. In this thesis, microsatellite markers on chromosome arms 3p, 4q, 8p, 9p, 11q, 13q and 17p were used, since they have been reported to lie within regions most frequently lost in oral SCCs. Each of these regions will be discussed briefly.

Chromosome 3:   High frequency of LOH at chromosome 3p has been reported in head and neck cancers (Table 1 in the appendix). The losses appear to center around 3p13-21.1, 3p21.3-23, and 3p24-25 (Maestro *et al.*, 1993; Partridge *et al.,* 1999, 1998; Partridge *et al.*, 1996; Roz *et al.*, 1996; Scully *et al.,* 1996). Studies have shown that LOH at 3p at early stage tends to be located in small regions and only at one of the 3 locus; whereas with progression of the carcinogenesis, a larger region of losses or loss at all three loci are noted at 3p. The increasing number of regions showing allele loss at 3p (3p 12.1-14.2, 21.3-22.1 and 24-26) is consistent with the progressive accumulation of genetic errors during the development of oral SCC (Partridge *et al.*, 1996).

Each of the three regions is presumed to contain at least one putative TSG. Within the region of 3p14.2 exists one of the most common fragile site locus, called FRA3B, in the human genome. Fragile sites are portions of chromosomes that are extremely weak and break easily. Consequently, these weak areas may be easy targets for carcinogens such as those found in tobacco. The gene, *FHIT* (fragile histidine triad), was

recently identified at this fragile site and appears to be involved in various cancers such as esophageal, gastric, colonic, breast, cervical, small cell lung, and head and neck carcinomas (Mao *et al.*, 1996a; Ohta *et al.*, 1996; Pennisi *et al.*, 1996; Sozzi *et al.*, 1996; Wilke *et al.*, 1996 and Wu *et al.*, 1994). It encodes a protein with 69% similarity to a *Schizosaccharomyces* pombe enzyme, diadenosine 5', 5'''-P1, P4-tetraphosphate (Ap4A) asymmetrical hydrolase which cleaves the AP4A substrate into 5' - ADP and AMP. Current theories suggest that diadenosine tetraphosphate may accumulate in the cells in the absence of the normal expression of the gene and may eventually lead to DNA synthesis and cell replication (Mao *et al.*, 1996).

Several recent studies have shown that *FHIT* may be significantly involved in oral SCC development (Croce *et al.*, 1999; Mao *et al.*, 1998; Tanimoto *et al.*, 2000; van Heerden *et al.*, 2001) and suggest that alteration to this gene may play an important role in the early stage of development of this cancer (Mao *et al.*, 1996). It was recently suggested in some tissues and organs, particularly those associated with exposure to environmental carcinogens, alterations in *FHIT* occur quite early in the development of human cancer (Croce *et al.*, 1999). Croce *et al.* concluded that *FHIT* loss in bronchial tissue indicates the occurrence of genetic alterations associated with the early steps of carcinogenesis. LOH at 3p14 has been shown to be involved in oral premalignant lesions (Mao *et al.*, 1996; Rosin *et al.*, 2000, 2002; Sukosd *et al.*, 2003; Uzawa *et al.*, 2001).

Until now there is sufficient evidence for only one gene, *FHIT*, to be responsible for the LOH at the region 3p14.3, although the evidence in support of it being a TSG is

still considered to be controversial (Mao *et al.*, 1998). TSGs that are responsible for LOH at the other two regions (3p24-pter, and 3p21.3) are still not clear. For example, the region of 3p24-25 contains the VHL gene, which is thought to be a member of a novel class of glycan-anchored membrane proteins that function in signal transduction and cell adhesion (Waber *et al.*, 1996), and its alteration has been reported especially in VHL-associated cancers (van den Berg *et al.*, 1997; Kok *et al.*, 1997 and Decker *et al.*, 1997). Uzawa *et al.* also mentioned the possibility that the VHL gene may in involved in oral SCC development (1998). However, mutations of the VHL gene could not be identified and the VLH gene was not inactivated by hypermethylation in HNSCC. Hypermethylation is an alternative method of inactivity of a gene that does not require direct mutation to the gene. It is possible that allelic loss of chromosome arm 3p in HNSCC involves regions surrounding the VHL locus but does not include the VHL gene. Another TSG in HNSCC may exist in the regions surrounding D3S 1110 at 3p 25 (Uzawa *et al.,* 1998; Waber *et al.*, 1996).

Chromosome 9:   LOH on 9p is by far the most commonly reported chromosomal defect in head and neck cancers, with LOH reported in 72% of malignant lesions. The most commonly affected region is chromosome 9p21-22. In addition, LOH at 9p22-q23.3 is also common (> 70% of head and neck cancers) (Lydiatt *et al.,* 1998 and Nawroz *et al.*, 1994). The putative TSGs are near the interferon locus and are not clearly identified.

At 9p21, the prime TSG candidate involved in head and neck cancers is *p16* (also know as *MTS-1* for major tumor-suppressor 1, *INK4a* for inhibitor of cyclin-dependent kinase4a, and *CDKN2A* for cyclin-dependent kinase inhibitor 2A). The TSG *p16 (INK4A/MTS-1/CDKN2A)* encodes a cell cycle protein that inhibits cyclin-dependent kinases (CDK) 4 and 6, preventing phosphorylation of Rb protein and consequently inhibiting the cell cycle transition of the G1-S phase (Reed *et al.*, 1996). The major biological effect of p16 is to halt cell-cycle progression at the G1/S boundary. The loss of p16 function may lead to cancer progression by allowing unregulated cellular proliferation (Stott *et al.*, 1998).

Although mutations of this gene are not apparently frequent in oral cancer, this might suggest that either this gene is inactivated by an alternative mechanism such as homozygous deletion or by methylation of the 5'CpG-rich region, which results in a complete block of gene transcription (Matsuda *et al.*, 1996; Merlo *et al.*, 1995; Papadimitrakopoulou *et al.*, 1997 and Rawnsley *et al.*, 1997). Reed *et al.* (1996) and Papadimitrakopoulou *et al.* (1997) found that ~80% of the head and neck cancers and premalignant lesions were p16 inactivated at the protein and/or DNA level and suggest that inactivation of p16 may play an important role in early head and neck cancer development. Alternatively, other tumor suppressor genes may exist in this region (Dawson *et al.*, 1996; Reed *et al.*, 1996 and Waber *et al.*, 1997).

Chromosome 17: LOH on 17p has been reported in 50% of head and neck cancers, most frequently involving 17p13 and 17p11.1-12 (Adamson *et al.*, 1994; Field *et*

al., 1996; Nawroz *et al.*, 1994). The region 17p13 harbors the gene *p53* (17p13.1), which has been reported to have the highest frequency (~50%) of mutations in human cancers. Mutation at p53 is also one of the most common events in HNSCC (Van Houten *et al.*, 2002). TP53 protein functions as mediator in several activities, including transcription activation, DNA repair, apoptosis, senescence, and G1/G2 cell cycle inhibition. In addition, increasing evidence has showed that a region, defined by the cholinergenic receptor B1 (CHRNB1) locus at 17p11.1-12, which is tightly linked to the *p53* regions, may contain a novel TSG.

Chromosome 4:   LOH on chromosome 4 has been studied in cancers of many systems and organs including hepatocellular, bladder, ovarian, and cervical cancers. The putative tumor suppressor locus was localized to a region near the epidermal growth factor gene on 4q25 and 4q24-26. Loss at 4q25 occurs in 70% of head and neck cancers (Pershouse *et al.*, 1997) and loss at 4q26-28 occurs in 47% (Bockmihl *et al.*, 1996; Califano *et al.*, 1996; Koch *et al.*, 1999). The combination of allelic deletions and chromosomal transfer studies strongly suggests the presence of a TSG within 4q24-26. This region was involved in >80% of the tumors examined, suggesting that a putative chromosome 4q TSG may play an important role in the evolution of HNSCC (Pershouse *et al.*, 1997).

Chromosome 8:   Investigation of 8p regions in head and neck squamous carcinoma has shown a relatively high incidence of alterations (31%-67%) (Ah-aee *et al.*, 1994; Bockmuhl *et al.*, 1996; Califano *et al.*, 1996; EI-Naggar *et al.*, 1995; Field *et al.*,

1995; Li *et al.*, 1994; Scholnick *et al.*, 1996 and Wu *et al.*, 1997). Deletion mapping of

oral and oropharyngeal SCC defines three discrete areas on chromosome arm 8p: 8p23,

8p22, and 8p12-p21 (EI-Naggar *et al.*, 1995; Ishwad *et al.*, 1999; Wu *et al.*, 1997).

Several studies have linked allelic loss at 8p to a higher stage (Wu *et al.*, 1997) and poor

prognosis (Li *et al.*, 1994 and Scholnick *et al.*, 1996).

Chromosome 11: LOH on human chromosome 11 has also been commonly

reported in a variety of human cancers, including HNSCC (39%-61%) (Bockmuhl *et al.*,

1996; Califano *et al.*, 1996; EI-Nagger *et al.*, 1995; Lazar *et al.*, 1998; Nawroz *et al.*,

1994; Uzawa *et al.*, 1996; Venugopalam *et al.*, 1998). The common region of loss at this

chromosome seems to be near the INT-2 locus at 11q13 (Nawroz *et al.*, 1994). It is

possible that some of this region's allelic imbalance may be due to amplification rather

than LOH (Nawroz *et al.*, 1994). Amplification of this region associated with poor

prognosis was also reported (Papadimitrakopoulou *et al.*, 1997).

Chromosome 13: More than half of HNSCCs shows LOH of 13q in regions

close to the RB (retinoblastoma) locus, but not *RB* gene (52-67%) (Bockmuhl *et al.*,

1996; Califano *et al.*, 1996; Maestro *et al.*, 1996; Nawroz *et al.*, 1994; Ogawara *et al.*,

1998). A hot spot of *D13s133* at 13q14.3, which lies just telomeric to the *RB* gene, was

reported (Yoo *et al.*, 1994). A recent study done by Ogawara *et al.* showed LOH on

13q14.3 correlated with lymph node metastasis of oral cancer ($P < 0.0024$). Their results

also suggest that LOH on 13q is a common event in oncogenesis and/or progression of

oral SCC and the existence of a new suppressor gene near *D13S273-D13S176* loci which

may play a role in these events since no significant variation in RB protein expression was detected (Ogawara *et al.,* 1998). The study of Harada *et al.* (1999) confirmed that LOH in chromosome 13 showed a significant correlation with lymph node metastasis in esophageal squamous cell carcinoma, as well as HNSCC. They reported that an unidentified TSG(s) in region 13q12-13 might be involved.

1.10 Molecular progression model for oral cancer

In the late 1980s, Fearon and Vogelstein, among the first people to describe molecular progression, suggested that a) tumors progress via the activation of oncogenes and the inactivation of TSGs, each generating a growth advantage for a clonal population of cells; b) specific genetic events generally occur in a distinct order of progression; but c) the order of progression is not necessarily the same for each individual tumor, and therefore it is the accumulation of genetic events that determines tumor progression. It has been estimated that at least 6-10 independent genetic events are required in order for head and neck cancers to occur (Emilion *et al.,* 1996). It is now accepted that the histologic progression of oral cancer (from hyperplasia → mild dysplasia → moderate dysplasia → severe dysplasia → CIS → SCC) is underlain by the accumulation of such changes to critical genes. In a landmark study by Califano and his colleagues (1996), LOH was investigated in a whole spectrum of oral premalignant lesions including hyperplasia, dysplasia, CIS, and SCC. The study proposed a genetic progression model for oral carcinogenesis. The model proposes that LOH at 9p is the earliest event associated with transition from normal to benign hyperplasia; LOH at 3p and 17p is

associated with dysplasia, whereas CIS and SCC were characterized by additional

deletions on 4q, 6p, 8p, 11q, 13q, and 14q.'

1.11 LOH in oral premalignant lesions

Since tumorigenesis is a sequential accumulation of genetic alterations, analysis

of early and late stage lesions may define the genetic changes associated with the

development and progression of HNSCC. Few studies have investigated the

premalignant stages of the lesions while there are many studies of LOH in oral SCC. The

main difficulties lie in the fact that: 1) premalignant lesions are small and therefore it is

extremely hard to obtain sufficient amount of DNA for molecular analysis, 2) big

hospitals or research centers typically have better access to cancers than premalignant

lesions, and 3) it is much harder to microdissect premalignant lesions compared to

carcinomas.

1.12 LOH as a risk marker for oral premalignant lesions

This lab has recently investigated the value of LOH as a risk marker for oral

premalignant lesions in a number of studies (Zhang *et al.*, 1997, 1999, 2000, 2001a, b,

2002; Rosin *et al.*, 2000, 2002; Poh *et al.*, 2001). In one study, we evaluated the use of

allelic loss to predict malignant risk for oral hyperplasia and low-grade oral epithelial

dysplasia. Two sample sets were used. The first set consisted of oral lesions from

patients with no subsequent history of head and neck cancer (non-progressing cases), which included 54 patients with biopsies of low-grade dysplasia (31 patients with mild dysplasia and 23 patients with moderate dysplasia) and 33 patients with epithelial hyperplasias. The second set (progressing cases) consisted of 29 patients (6 hyperplasias, 9 mild dysplasias, and 14 moderate dysplasias) that later progressed to CIS and SCC. Each of the 116 cases was analyzed for LOH at 19 microsatellite loci on seven chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q, and 17p).

LOH frequencies were dramatically elevated in lesions that later progressed to cancer. All (100%) progressing lesions (both hyperplastic and dysplastic) showed LOH at one or more of the 19 microsatellite loci tested for the 7 chromosome arms. In contrast, LOH was detected in only 21% of the non-progressing hyperplasias and 59% of non-progressing dysplasias.

Multiple chromosomal arm loss was characteristic of progressing lesions (50% of hyperplasia and 91% of dysplasia). It was absent in non-progressing hyperplasia and occurred in only 31% of the non-progressing dysplasias.

The progressing and non-progressing cases showed different LOH patterns of multiple allelic losses. Three risk groups were deduced based on genetic profile: a low-risk LOH pattern (retention of 3p and 9p); an intermediate-risk (loss at 3p and/or 9p) and high-risk pattern (loss at 3p and/or 9p plus loss at 4q, 8p, 11q, 13q or 17p). Leukoplakia with LOH at sites on 3p and/or 9p had a 3.8-fold increase in relative risk of developing

cancer. Lesions with additional loss on other chromosome arms had a 33-fold increase in cancer risk as compared to those that retained 3p and 9p (Rosin *et al.*, 2000; editorial on the article by Mao, 2000). The study indicates that employment of microsatellite analysis of LOH could identify premalignant lesions with high cancer risk even though they are histologically low grade.

In a more recent study from our lab, we investigated the use of LOH markers to predict the risk of developing a second oral malignancy (SOM). A total of 68 leukoplakias at former cancer sites with known outcome were evaluated for the loss of heterozygosity at 19 loci on seven chromosome arms. Thirty six of the 68 cases progressed to SOM later. It was found that 3p and/or 9p loss in these post-treatment leukoplakia was associated with a 26.3-fold increase in risk of developing SOM compared with those that retained both of these arms ($P < 0.001$), with 60% of cases developing SOM in 2 years. This study had shown that the identification of 3p and 9p loss in post-treatment lesions could serve as a simple and direct test for stratifying risk of SOM development (Rosin *et al.*, 2002).

This thesis will employ similar molecular markers on the 7 chromosome arms studies to compare the genetic risk of low-grade dysplasia adjacent to oral SCC and those of the primary oral SCC.

2 Statement of the problem

Surgical excision of oral SCC currently involves removal of at least 1 cm of normal looking oral mucosa outside of the clinically visible tumors, if possible. The excisional samples are judged histologically for the margin. Currently if tumor or high-grade dysplasia (e.g. severe dysplasia) has involved the resection margin, aggressive treatment will be employed to eradicate the cancerous or high-grade dysplastic changes. However, when low-grade dysplasia has involved the biopsy margin, there is no general agreement on whether further treatment is needed. Therefore, many of these lesions are left untreated. It is not clear whether these low-grade dysplastic changes have higher cancer risk than those morphologically similar but from patient without a history of cancer, which have been shown to be of low cancer risk. Studies are needed to address this important question. If these low-grade lesions are of high cancer risk they may contribute to the high recurrence of oral SCC, if left untreated.

3 Objectives

1. To obtain information on the pattern of genetic changes in epithelial hyperplasia, mild epithelial dysplasia and moderate dysplasia adjacent to SCC or CIS, by means of LOH analysis using microsatellite markers for the 7 chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q and 17p).

2. To obtain information on the pattern of genetic changes of primary epithelial hyperplasia, mild epithelial dysplasia, and moderate dysplasia by means of LOH analysis using microsatellite markers for the 7 chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q and 17p).

3. To determine whether LOH profile was significantly altered in low-grade lesions (hyperplasia or low-grade dysplasia) taken from adjacent to SCC/CIS as compared to morphologically similar primary lesions.

4 Hypothesis

Low-grade dysplasias (mild/moderate) and hyperplasias adjacent to SCC or CIS contain more high-risk LOH pattern than primary low-grade dysplasia and hyperplasia.

5   Materials and Methods

5.1   *Sample collection*

This study used paraffin-embedded archival samples from the provincial Oral

Biopsy Service of British Columbia. This centralized Oral Biopsy Service provides

service to dentists and ENT surgeons throughout the province, at no cost to the provider

or patient, with more than 3,500 biopsies of oral lesions received per year (23 years

archived). This provides a large collection of early lesions that can be followed over

time. Cases that progressed into cancer were identified by linking the database of this

Service to the British Columbia Cancer Registry, which receives notification of all

histologically confirmed cases of cancer and CIS diagnosed in the Province. All primary

lesions used in this study were checked against the BCCA registry to preclude

progression into cancer or history of previous cancer.

5.2   *Study groups*

There are 4 groups of patients in this study. The first group consists of 30 cases

of primary hyperplasia. The second group consists of 45 cases of primary low-grade

dysplasia. The third group consists of 15 cases of hyperplasia taken from adjacent to

SCC or CIS. The fourth group consists of 38 low-grade dysplasias taken from adjacent to SCC or CIS. An summary:

Group 1:   30 primary hyperplasia.

Group 2:   45 primary low-grade dysplasia (mild/moderate).

Group 3:   15 hyperplasia taken from adjacent to oral SCC or CIS.

Group 4:   38 low-grade dysplasia (mild/moderate) taken from adjacent to oral SCC or CIS.

5.3   *Histological diagnostic criteria for the samples*

The criteria for choosing samples included:

1)   A histological diagnosis of a case confirmed by two pathologists using criteria established by the World Health Organization (WHO collaborating Reference centre 1978).

2)   The provision that the sample was large enough to yield sufficient DNA from both the epithelium and from the connective tissue for multiple LOH analyses.

The histological diagnoses of the lesions were performed independently by Dr. R. Priddy and Dr. L. Zhang, oral pathologists at the University of British Columbia. Only those cases in which the two pathologists agreed on the diagnosis were used for the study.

5.4   *Patient information*

In addition to histological diagnosis of the lesions, the following patient information was collected: age, gender, site of the lesion, history of cancer, and smoking habits.

5.5   *Slide preparation*

Following confirmation of diagnosis and sufficient tissue on blocks, a 5 micron section was cut from each block and stained with hematoxylin and eosin (H&E) for use as a dissection reference slide. Further 12 micron thick sections were cut and put on glass slides for dissection. These slides were also stained with H&E. The H&E procedure is described below:

The sections were baked overnight at $37\,^{\circ}C$ in an oven, then at $60\text{-}65\,^{\circ}C$ for 1 hour, and were left at room temperature to cool. Samples were deparaffinized by two changes of xylene for 15 minutes each, then the xylene was cleared by graded ethanol (100%, 95% and 70%), and hydrated by rinsing in tap water. Slides were then placed in Gill's Hematoxylin for 5 minutes, followed by rinsing in tap water, and were then blued with 1.5% (w/v) sodium bicarbonate. After rinsing in water, the H.E. slides were lightly counterstained with eosin, dehydrated, and cleared for coverslipping. Thick sections to be dissected were stained by the above procedure without the dehydration step, and air dried (Michelsen, 1997).

5.6   *Tissue microdissection*

Areas of hyperplasia and dysplasia were microdissected from sections stained with hematoxylin/eosin. DNA from tissues were obtained by dissecting out the underlying stroma in these sections and used as control DNA for the case. Under a dissection microscope, the dysplastic or hyperplastic epithelial cells were separated from underlying connective tissues with a 1 ml syringe needle. The collected connective tissue was used as a control for the study, as the epithelium tissue was used for experiment. The tissues were put in a 1.5 ml eppendorf tube separately.

5.7   *Sample digestion and DNA extraction*

The microdissected tissue was placed in an Eppendorf tube and digested in 300 μl of 50 mM Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and proteinase K (0.5mg/ml) at a 48°C water bath for 72 or more hours. During incubation, samples were spiked with 10 - 20 μL of fresh concentrated proteinase K (20 mg/ml) twice daily.

The DNA was then extracted two times with PC-9, a phenol-chloroform mixture, and precipitated with 100% ethanol in the presence of glycogen. DNA was resuspended in Tris buffer.

5.8   *DNA quantification*

Sample DNA was quantified with fluorescence analysis using Picogreen kit (Molecular Probes). The absorbance was read from an SLM 4899C spectrofluorometer. The amounts of sample DNA were determined from the standard curves which were made by the absorbance of known concentration of standard DNA provided by the Picogreen kit. The calibration with known concentration of DNA with standard curve was repeated in every experiment to ensure reproducibility.

5.9   *LOH analysis*

The 19 microsatellite markers were used for LOH analysis came from Research Genetics (Huntsville, AL) and they were mapped to the following regions: 3p14.2 (*D3S1234, D3S1228, D3S1300*); 4q26 (*FABP2*); 4q31.1 (*D4S243*); 8p21.3 (*D8S261*); 8p23.3 (*D8S262, D8S264*); 9p21 (*IFNA, D9S171, D9S1748, D9S1751*); 11q13.3 (*INT2*); 11q22.3 (*D11S1778*); 13q12.3-13 (*D13S170*); 13q14.3 (*D13S133*); 17p11.2 (*CHRNB1*) and 17p13.1 (*tp53* and *D17S786*). These markers are localized to regions previously shown to be frequently lost in head and neck tumors (Bockmihl *et al*, 1996; Califano *et al.*, 1996, 1999, 2000; Koch *et al.*, 1999; Lydiatt *et al.,* 1998; Nawroz *et al.*, 1994; Partridge *et al.,* 1996, 1999, 1998; Van Houten *et al*, 2002; Uzawa *et al.*, 1996). The protocol used for LOH analysis is described in Zhang *et al.*, 1997.

5.9.1 End-labeling

The reaction was performed in a 50 µl mixture containing the following: 38 µl of PCR-quality distilled water, 5 µl of a 10 × buffer for T4 polynucleotide kinase (New England BioLabs: Ontario), 1 µl of 10 × Bovine Serum Albumin, 1 µl of one of the primer pairs, 3 µl of T4 polynucleotide kinase (New England BioLabs: Ontario), and 2 µl of [γ-^{32}P] ATP (20 µCi, Amersham: NJ, USA). The labeling was done in a single reaction in the thermocycler at 37°C for 60 min (Michelsen 1997).

5.9.2 PCR Amplification for microsatellite analysis

The PCR amplification was carried out in a 5 µl reaction volume containing 5ng of genomic DNA, 1ng of labeled primer, 10 ng of each unlabeled primer, 1.5 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 units of Taq DNA polymerase (Life Techs: Ontario), PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH8.8), 6.7 mM magnesium chloride, 10mM β–mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide], and 2 drops of mineral oil. The amplification reaction was run in the thermal-cycler for 1 cycle of pre-heat at 95°C for 2 min; 40 cycles of denaturation at 95°C for 30s, annealing at 50-60 °C (depending on the primer used) for 60s, and polymerization at 70 °C for 60 sec; followed by 1 cycle of final polymerization at 70 °C for 5 min.

After PCR amplification, PCR products were separated on 7% urea-formamide-polyacrylamide gels and visualized by autoradiography. For informative cases, allelic loss was inferred when the signal intensity of one allele was decreased by at least 50% in the DNA sample from a lesion, as compared to the corresponding allele in the matching connective tissue DNA. Samples showing allelic loss were subjected to repeat analysis after a second independent amplification whenever the quantity of DNA was sufficient.

5.9.3 Primer-Extension Preamplification (PEP)

In several cases, DNA concentrations were too low to perform multiple LOH assays. To increase the amount of available DNA, these samples were subjected to total genomic DNA amplification prior to LOH assay, using a PCR-based technique developed by Zhang *et al.*, 1997, 10 ng of DNA was suspended in 60 μl reaction mixtures containing 90 mM Tris-HCl, pH 8.3, 33 μM random 15-mer primer (Operon Techs., SP180-2), 5 units Taq DNA polyermase (Gibco BRL), 100 μM each of dATP, dGTP, dCTP, and dTTP, and PCR buffer [2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 100 μg/ml gelatin (Fisher G8-500)]. PCR amplification was performed for 50 cycles consisting of denaturation at 92°C for 1 minute, annealing at 37°C for 2 minutes, ramping at 10sec/degree to 55°C and a final extension at 55°C for 4 minutes. Two μl aliquots from this reaction were then assayed for the presence or absence of LOH using a microsatellite based-PCR assay.

5.10 Statistical analysis

Differences and associations between hyperplasia/dysplasia from cancer patients and primary hyperplasia/dysplasia were examined using either Fisher's exact test for categorical variables (gender, smoking habit, and LOH) or t-test for continuous variables (age). All tests were two sided. $P < 0.05$ was considered to be statistically significant and those less than 1 considered approaching significant or marginally significant.

6 Results

6.1   Demographic information

In this study, 128 cases were studied, including 30 primary hyperplasia, 45 primary low-grade dysplasias, 15 hyperplasias taken from adjacent to oral cancer, and 38 low-grade dysplasias taken from adjacent to oral cancer. Table 1 summarizes the demographic characteristics of all cases in this study. Patient ages ranged from 16 to 91 years, with a mean of 54 and standard deviation of 17. Fifty five percent were male, and sixty percent from the study population had a smoking habit (ever-smoker).

Cases with primary lesions (hyperplasia and low-grade dysplasia) and lesions (hyperplasia and low-grade dysplasia) adjacent to SCC/CIS were compared for the aforementioned clinicopathological features. No significant association was observed between age, gender, or smoking history (Table 1, all have $P > 0.05$).

Of the 75 primary lesions, 39 cases (52%) came from high-risk sites (tongue, floor of mouth and the soft palate complex), and 38 cases (48%) were from low-risk sites (the rest of oral cavity). In the 53 lesions taken from adjacent to cancers, 42 (79%) were from high-risk sites and only 11 (21%) came from low-risk sites. The primary lesions showed significantly fewer cases located in the high-risk region ($P = 0.002$)

Table 1. Demographic features of primary lesions and lesions adjacent to cancers

	All cases	Primary lesions (%)	Lesions adjacent to cancer (%)	P value*
Total	128	75 (59%)	53 (41%)	
Mean age (yr) ± SD	54 ± 17	55 ± 16	53 ± 17	0.52
Male sex -- no. (%)	67/126 (53%)	39/73 (53%)	28/53 (53%)	1.00
Ever smoker -- no. (%)	59/99 (60%)	37/59 (63%)	22/40 (55%)	0.53
Histology *Hyperplasia* *Low-grade dysplasia*	45 83	30 45	15 38	0.19
Lesions on ventrolateral tongue, floor of mouth & soft palate complex (%)	81/128 (63%)	39/75 (52%)	42/53 (79%)	**0.002**

*P-values between primary lesions and lesions adjacent to cancer.

The lesions were then separated according to the histological diagnosis. Table 2 demonstrates the demographic information for all hyperplasias, and also compares the primary hyperplasia with hyperplasia taken from adjacent to cancer. The age, gender, or smoking histories between the two groups were similar and no significant difference was found (all have $P > 0.05$). However, a significantly higher proportion of hyperplasias taken adjacent to cancer were from the ventrolateral tongue and floor of mouth region ($P = 0.004$).

Table 2. Demographic features of hyperplastic lesions

	All hyperplasias	Primary hyperplasia (%)	Hyperplasia adjacent to cancer (%)	P value[*]
Total	45	30	15	
Mean age (yr) ± SD	53 ± 3	52 ± 3	54 ± 5	0.82
Male sex -- no. (%)	29/45 (64%)	18/30 (60%)	11/15 (73%)	0.51
Ever smoker -- no. (%)	22/39 (56%)	14/26 (54%)	8/13 (62%)	0.74
Lesions on ventrolateral tongue, floor of mouth & soft	21/45 (47%)	9/30 (30%)	12/15 (80%)	**0.004**

*P-values between primary hyperplasia and hyperplasia adjacent to cancer.

Table 3 demonstrates the demographic information for all low-grade dysplasias and compares the primary low-grade dysplasia with the low-grade dysplasia taken from adjacent to cancer. There were no differences in the age, gender, smoking history or the site of the lesions (all have $P > 0.05$).

Table 3. Demographic features of low-grade dysplasias

	All dysplasias	Primary low-grade dysplasia (%)	Low-grade dysplasia adjacent to cancer (%)	P value[*]
Total	83	45	38	
Mean age (yr) ± SD	55 ± 2	57 ± 2	53 ± 3	0.24
Male sex -- no. (%)	38/81 (47%)	21/43 (48.8%)	17/38 (44.7%)	0.82
Ever smoker -- no. (%)	37/60 (62%)	23/33 (69.7%)	14/27 (51.9%)	0.19
Lesions on ventrolateral tongue, floor of mouth & soft palate complex (%)	60/83 (72%)	30/45 (67%)	30/38 (79%)	0.23

P-values between primary low-grade dysplasia and low-grade dysplasia adjacent to cancer.

The demographic results show that age, gender, and smoking history were not confounders and did not introduce biases to the experiment. While the site of the lesions was not a confounder for the dysplastic lesions, it is a confounder for the hyperplastic lesions as primary hyperplastic lesions were more likely to be located at the low-risk region.

6.2   *LOH and histology*

Table 4 compares LOH frequencies in hyperplasia and low-grade dysplasias. Consistently higher rates of LOH at all categories were observed in lesions with dysplasia as compared to non-dysplastic lesions, and the differences in almost all of the categories were significant.

When the number of losses was examined, dysplasias showed significant increases in LOH as compared to hyperplasias for any losses (66% vs. 11%, P < 0.0001), for more than 1 arm lost (37% vs. 2%, P < 0.0001), and for more than two arms lost (29% vs. 2%, P < 0.0001).

For the LOH patterns of individual chromosome arms, the increases in LOH for dysplasia as compared to hyperplasia were very significant for 3p loss (25% vs. 4.5%, P = 0.006), 4q loss (20% vs. 0%, P = 0.004), 8p loss (25% vs. 5%, P = 0.02), 9p loss (44% vs. 4.5%, P < 0.0001), 11q loss (22% vs. 2%, P = 0.004), 13q loss (18% vs. 2%, P = 0.03), and 17p loss (30% vs. 2%, P < 0.0001).

When the pattern was inspected, LOH at 3p and /or 9p loss (52% vs. 2%, P < 0.0001) and LOH at 3p and/or 9p plus 17p lost (35% vs. 2%, P < 0.0001) were significantly higher in dysplastic lesions, compared to hyperplasias.

It is clear that lesions with dysplastic changes have much higher LOH frequencies in all chromosome regions tested than hyperplasia.

Table 4. LOH pattern between hyperplasia and low-grade dysplasia

	Lesions with hyperplasia	Lesions with dysplasia	P value [a]
# of lesions	45	83	
# with LOH [b]	5/45 (11%)	55/83 (66%)	< 0.0001
>1 arm lost	1/45 (2%)	31/83 (37%)	< 0.0001
>2 arms lost	1/45 (2%)	24/83 (29%)	< 0.0001
LOH on: 3p	2/44 (4.5%) [c]	20/80 (25%)	0.006
4q	0/37 (0%)	13/66 (20%)	0.004
8p	2/38 (5%)	17/67 (25%)	0.02
9p	2/44 (4.5%)	35/80 (44%)	< 0.0001
11q	1/43 (2%)	15/67 (22%)	0.004
13q	1/42 (2%)	11/61 (18%)	0.03
17p	1/44 (2%)	24/81 (30%)	< 0.0001
3p &/or 9p	1/45 (2%)	43/82 (52%)	< 0.0001
3p &/or 9p plus others	1/45 (2%)	29/82 (35%)	< 0.0001

[a] *P-values* between lesions with hyperplasia and dysplasia.
[b] A total of seven chromosomal arms were tested.
[c] Loss/ informative cases (%).

6.3   *LOH and presence of cancer*

LOH frequencies were compared between primary hyperplasia/low-grade dysplasia and those morphologically similar lesions taken from adjacent to oral cancer (Table 5). Consistently higher rates of LOH at all categories were observed in lesions adjacent to SCC/CIS as compared to the primary lesions and the differences in almost all of the categories were statistically significant.

When the number of losses was examined, lesions taken from adjacent to cancer showed increased LOH frequency in any loss as compared to the primary lesions (57% vs. 40%, $P = 0.07$), and an extremely significant increase for > 1 arm loss (41.5% vs. 13%, $P = 0.0004$), and for more than 2 arms losses (38% vs. 7% in patients, $P < 0.0001$).

For individual chromosome arms, the increase in LOH for lesions taken adjacent to cancer as compared to primary lesions was very significant for 4q loss (24% vs. 3.5%, $P = 0.002$), 11q loss (25.5% vs. 5%, $P = 0.003$), 13q loss (22% vs. 4%, $P = 0.006$), and 9p loss (41.5% vs. 21%, $P = 0.02$). An increased frequency in LOH was noted for 3p (25% vs. 12.5%, $P = 0.10$), and 17p (28% vs. 14%, $P = 0.07$). The only category obviously not statistically significant was 8p loss (23% vs. 14%, $P = 0.31$).

When the pattern of LOH was considered, LOH for 3p and/or 9p (48% vs. 25%, $P = 0.01$), and LOH for 3p and/or 9p plus 17p (42% vs. 11%, $P < 0.0001$) were significantly higher in lesions taken from adjacent to cancer as compared to primary lesions.

Table 5. LOH frequencies primary lesions and lesions taken from adjacent to cancer

	Primary hyperplasia and low-grade dysplasia	Hyperplasia and low-grade dysplasia adjacent to cancer	P value[a]
# of lesions	75	53	
# with LOH [b]	30/75 (40%)	30/53 (57%)	0.07
>1 arm lost	10/75 (13%)	22/53 (41.5%)	0.0004
>2 arms lost	5/75 (7%)	20/53 (38%)	< 0.0001
LOH on: 3p	9/72 (12.5%)[c]	13/52 (25%)	0.10
4q	2/57 (3.5%)	11/46 (24%)	0.002
8p	8/57 (14%)	11/48 (23%)	0.31
9p	15/71 (21%)	22/53 (41.5%)	0.02
11q	3/59 (5%)	13/51 (25.5%)	0.003
13q	2/56 (4%)	10/46 (22%)	0.006
17p	10/72 (14%)	15/53 (28%)	0.07
3p &/or 9p	19/75 (25%)	25/52 (48%)	0.01
3p &/or 9p plus others	8/75 (11%)	22/52 (42%)	< 0.0001

[a] P-values between lesions with hyperplasia and dysplasia.
[b] A total of seven chromosomal arms were tested.
[c] Loss/ informative cases (%).

6.4   *LOH pattern and presence of SCC/CIS in hyperplastic lesions*

Since the proportion of hyperplasia and low-grade dysplasia in the primary groups and cancer groups differed, and the cancer group had a higher proportion of dysplasia, there is a possibility that the increase in the LOH seen in the cancer group resulted from increased number of dysplasia. To rule out the possibility, primary and cancer lesions of different histology were separated.

Table 6 compared LOH patterns in primary hyperplasias and hyperplasias taken adjacent to cancer. LOH was observed in low frequency in both groups of hyperplasias. There was no significant difference in all categories of LOH frequencies between primary hyperplasia and those histologically similar hyperplasia adjacent to SCC/CIS: cases with any LOH (13% vs. 7%, $P = 0.65$), more than 1 arm lost (0% vs. 7%, $P = 0.33$), more than 2 arms lost (0% vs. 7%, $P = 0.33$), LOH on 3p (3% vs. 7%, $P = 1.00$), 4q loss (0% vs. 0%, $P = 1.00$), 8p (4% vs. 8%, $P = 0.32$), 9p (3% vs. 7%, $P = 1.00$), 11q (0% vs. 7%, $P = 0.33$), 13q (0% vs. 8%, $P = 0.32$), and 17p (3% vs. 0%, $P = 1.00$), and LOH for 3p and/or 9p loss (0% vs. 7%, $P = 0.333$), and for 3p and/or 9p plus 17p loss (0% vs. 7%, $P = 0.33$).

Table 6. LOH pattern between primary hyperplasia and hyperplasia adjacent to SCC/CIS

	Primary hyperplasia	Hyperplasia adjacent to cancer	P value [a]
# of lesions	30	15	
# with LOH [b]	4/30 (13%)	1/15 (7%)	0.65
>1 arm lost	0/30 (0%)	1/15 (7%)	0.33
>2 arms lost	0/30 (0%)	1/15 (7%)	0.33
LOH on: 3p	1/29 (3%) [c]	1/15 (7%)	1.00
4q	0/25 (0%)	0/12 (0%)	1.00
8p	1/25 (4%)	1/13 (8%)	0.32
9p	1/29 (3%)	1/15 (7%)	1.00
11q	0/29 (0%)	1/14 (7%)	0.33
13q	0/28 (0%)	1/13 (8%)	0.32
17p	1/29 (3%)	0/15 (0%)	1.00
3p &/or 9p	0/30 (0%)	1/15 (7%)	0.33
3p &/or 9p plus others	0/30 (0%)	1/15 (7%)	0.33

[a] P-values between lesions with hyperplasia and dysplasia.
[b] A total of seven chromosomal arms were tested.
[c] Loss/ informative cases (%).

6.5   *LOH pattern and presence of SCC/CIS in low-grade dysplasias*

Table 7 compared LOH patterns in primary low-grade dysplasias and those morphologically similar lesions taken from adjacent to oral cancer. Consistently higher rates of LOH in all categories were observed in low-grade dysplasias taken adjacent to SCC/CIS as compared to the primary low-grade dysplasias and the differences in the majority of the categories were significant.

When the number of losses was examined, compared to the primary low-grade dysplasias, low-grade dysplasia taken from adjacent to cancer showed a significant increase for LOH at more than 1 arm losses (55% verses 22%, $P = 0.003$) and for more than two arm losses (50% vs. 11%, $P = 0.0002$), although no statistical difference was noted for any loss (76% vs. 58%, $P = 0.10$).

LOH on individual chromosome arms in low-grade dysplasias adjacent to tumor compared to primary low-grade dysplasia were 32% vs.19% for 3p ($P = 0.20$), 32% vs. 6% for 4q ($P = 0.01$), 29% vs. 22% for 8p ($P = 0.58$), 55% vs. 33% for 9p ($P = 0.07$), 32% vs. 10% for 11q ($P = 0.04$), 27% vs. 7% for 13q ($P = 0.51$), and 39.5% vs. 21% for 17p ($P = 0.09$). The differences were statistically significant for 4q and 11q ($P = 0.01$ and 0.04 respectively).

For LOH patterns, the difference was significant for 3p and/or 9p (65% vs. 42.2%, P = 0.05) and for 3p and/or 9p plus other arms (57% vs.18%, P = 0.0004).

These results indicate that the differences in LOH results between primary lesions (both hyperplasia and low-grade dysplasia) and those taken from adjacent to cancer resulted from the differences between the dysplastic lesions and not the hyperplastic lesions.

Table 7. LOH pattern between primary low-grade dysplasia and low-grade dysplasia adjacent to SCC/CIS

	Primary low-grade dysplasia	Low-grade dysplasia adjacent to cancer	P value[a]
# of lesions	45	38	
# with LOH[b]	26/45 (58%)	29/38 (76%)	0.10
>1 arm lost	10/45 (22%)	21/38 (55%)	**0.003**
>2 arms lost	5/45 (11%)	19/38 (50%)	**0.0002**
LOH on: 3p	8/43 (19%)[c]	12/37 (32%)	0.20
4q	2/32 (6%)	11/34 (32%)	**0.01**
8p	7/32 (22%)	10/35 (29%)	0.58
9p	14/42 (33%)	21/38 (55%)	**0.07**
11q	3/30 (10%)	12/37 (32%)	**0.04**
13q	2/28 (7%)	9/33 (27%)	0.51
17p	9/43 (21%)	15/38 (39.5%)	**0.09**
3p &/or 9p	19/45 (42%)	24/37 (65%)	**0.05**
3p &/or 9p plus others	8/45 (18%)	21/37 (57%)	**0.0004**

[a] P-values between lesions with hyperplasia and dysplasia.
[b] A total of seven chromosomal arms were tested.
[c] Loss/ informative cases (%).

7 Discussion

Oral cancer has a high recurrence rate. About one third of oral SCC will recur and the majority of this recurrence occurs within 2 years of the treatment of the primary cancer. The tumor recurrence is usually attributed to the outgrowth of residual malignant or adjacent high-risk premalignant cells left behind in the margins of treated lesions.

While high-grade premalignant lesions at the margins of the oral cancer are regarded as high-risk and will invoke aggressive salvage treatment, currently there is no agreement on whether low-grade lesions left at the tumor margins should be treated. Clinicians may feel reluctant to go back for further salvage treatment when the margins are involved with low-grade dysplasia. Primary low-grade dysplasias are known to have a low risk for cancer transformation; however, it is not clear whether low-grade dysplasia at the margin of cancer also has low cancer risk.

One way of assessing the cancer risk of low-grade dysplasia at the margins of oral tumors is by assessing the molecular risk of these lesions. Recent studies have shown that molecular markers could be used to identify high-risk OPL. According to a previous study from our lab, primary low-grade dysplasias which contain LOH on 3p and/or 9p plus other chromosome arms has a 33-fold increased risk of progressing into cancer later (Rosin *et al.*, 2000 and Zhang *et al.*, 2000). A more recent study from our lab has shown that, for patients with a history of oral cancer, microsatellite markers can also be used to

predict cancer risk of leukoplakias at the index tumor sites (Rosin *et al.*, 2002). 3p &/or 9p loss in the post-treatment leukoplakia was associated with a 26.3-fold increase in risk of developing into cancer compared to those that retained both of these arms ($P < 0.001$), with 60% of cases with LOH developing cancer in 2 years.

This study investigated the cancer risk of low-grade dysplasias adjacent to cancers. The results could be used to improve the management of the surgical margins of oral cancer.

7.1   *Presence of dysplasia signals increased molecular risk*

When dysplastic lesions from both patients without a history of oral cancer and patients with a history of oral cancer were compared to nondysplastic lesions, a markedly increased LOH frequency was noted in all categories examined (Table 4), including multiple losses and LOH at 3p and/or 9p plus other arms, a pattern shown to associate with a 33-fold increase in relative cancer risk. Such data are consistent with those of the literature (Mao *et al.,* 1996a; Partridge *et al.,* 1999, 1998; Poh *et al.*, 2001; Rosin *et al.*, 2000, 2002; Zhang *et al.*, 1997, 1999, 2000, 2001a, b, 2002) including the results from our previous studies that demonstrated the increased molecular risk associated with the dysplastic phenotype and supported the value of the gold standard histology.

7.2   *Hyperplasia adjacent to cancer showed no increased molecular risk*

The significance of the phenotype of dysplasia is further supported by the data that hyperplasia adjacent to SCC/CIS showed no significant increase in LOH when compared with primary hyperplasia (Table 6). The frequencies of LOH were very low in both primary hyperplasias and hyperplastic epithelial tissue taken from the margin of the tumors, even though hyperplastic tissues taken from adjacent to cancer were more likely to be at the high risk region. The results reinforce the significant role of the gold standard histology in the assessment of cancer risk of tumor margins. Interestingly one of the hyperplasias taken from the margins of the tumor resection samples showed a high-risk molecular pattern, suggesting that this case has increased cancer risk despite the absence of apparent dysplasia.

7.3   *Low-grade dysplasias adjacent to cancer have increased molecular risk*

In sharp contrast to hyperplasias, a striking difference in LOH frequencies was observed between primary low-grade dysplasias and those morphologically similar lesions taken from adjacent to cancer (Table 7).

Low-grade dysplasias adjacent to oral cancers had a significant increase in multiple LOH as compared to the primary low-grade dysplasia: the LOH frequency had more than doubled for more than 1 loss (55% vs. 22%, $P = 0.0029$) and had increased almost five times for more than 2 losses (50% vs. 11%, $P = 0.0002$). Similarly the high-risk LOH patterns were observed significantly more frequent in low-grade dysplasias adjacent to cancers as compared to the primary low-grade lesions: the LOH frequency of 3p and/or 9p was higher in the cancer group ($P = 0.0483$), and 3p and/or 9p plus other arms increased more than 3 times from 18% in primary low-grade dysplasias to 57% in low-grade dysplasia adjacent to SCC/CIS ($P = 0.0004$). Both multiple losses and LOH pattern of 3p and/or 9p plus other arms have been found to correlate with markedly increased cancer risk by our research team and by other laboratories (Califano *et al.*, 1996; Mao *et al.*, 1996; Partridge *et al.*, 2000; Rosin *et al.*, 2000, 2002).

The LOH frequencies on individual arms also differed. Low-grade dysplasia adjacent to cancer had a significant increase in 4q and 11q losses. Our previous studies showed that LOH at 4q and 11q are rare in primary low-grade dysplasia but significantly increased in high-risk lesions such as verrucous hyperplasia and severe dysplasia, or low-grade lesions that later progressed into cancer (Poh *et al.*, 2000 and Rosin *et al.*, 2000). All of these suggest that low-grade dysplasia adjacent to cancer should not be regarded in general as low-risk lesions, as are morphologically similar primary lesions. This study may suggest a more careful follow-up for patients with residue low-grade dysplasia in the surgical margins.

While dysplasias taken from adjacent to cancer have an overall higher molecular risk as compared to those morphologically similar primary lesions, the results also showed that about half or a little less than half of the low-grade dysplasias adjacent to cancer were molecularly low risk judged by multiple losses (45% had not demonstrated > 1 arm loss and 50% had not demonstrated > 2 arm losses) or by LOH at 3p & 9p plus other chromosome arms (43% did not have such loss). These results again illustrate the value of molecular markers in triage lesions of different risks that allow aggressive treatment of those low-grade dysplasias with high molecular risk to prevent tumor recurrence on the one hand but avoid unnecessary aggressive or mutilating surgery on the other.

7.4 *Limitations of the study and future plan*

This study has shown markedly increased molecular risk for many low-grade dysplasias taken from adjacent to cancers. Since increased molecular risk has been shown to be significantly associated with increased cancer risk, such results would suggest aggressive salvage treatment for patients with such residual low-grade dysplasia. This would be a significant alteration to the current practice since, in many cases, the residual dysplasia is not removed subsequent to the histological evaluation after treatment.

However, before the results can be applied clinically and affect patient management, the study data must be further tested and confirmed because of the limitation of the current study.

It is possible that the study results were biased by the small sample set that was used. Because of time restraint for a MSc thesis and manpower limitations for microdissection and multiple primer analysis of samples, I could only manage 128 samples. Larger sample numbers, preferably from different study centers, will be needed to confirm the study results.

Although microsatellite analysis is currently one of the most sensitive molecular techniques for assaying small samples, it still requires at least 100 ng of DNA for multiple primer analysis. Consequently, the selection of samples was biased by the preferential selection of larger oral premalignant lesions with sufficient amount of DNA from both the epithelium and the connective tissue; those samples with less than 100 ng of DNA were excluded. Advancement in technology sensitivity that would allow analysis of much smaller amount of samples will be needed to eliminate this bias.

A major limitation of the study comes from the fact that this study has a retrospective design. It is well known that retrospective studies are inherently prone to bias. Without exhausting the many possible biases and partialities associated with retrospective studies, I will list some of the limitations of retrospective studies for my study.

The information obtained from retrospective studies is frequently inaccurate or lacking and hence may miss confounding factors (that could produce false associations in parameters). Patients with complete pathological records were preferentially selected rather than those that had missing information, hence producing sample selection bias.

Of all the limitations that are associated with retrospective studies, the most significant limitation is that it is not clear what the relationship was between the 'adjacent dysplasias' from cancer samples and the surgical margins of the cancers. Ideally, one would like to know if the adjacent dysplasia analyzed for loss of heterozygosity has involved the biopsy margins. Theoretically, if the molecularly high-risk lesions were left behind after treatment, these lesions would have higher risk of developing into recurrence compared to morphologically similar lesions without the high-risk molecular pattern. However, if the samples from the high-risk low-grade lesions did not involve the biopsy margins, its high-risk would not affect the outcome. Since my data lacked information on whether or not the sample involved the biopsy margins, I am not able to correlate the data with the outcome, the gold standard for validating any biomarkers. I also do not know whether further treatment (surgery) was given to these cases, to increase the margin width. Such treatment would also affect outcome.

For future plans, a large-scale prospective study will be needed to confirm the study results. Ideally, low-grade dysplasia for molecular analysis should be taken from the margin of the cancer excision sample. Longitudinal study will allow comparison

between the outcomes of patients with residual high molecular risk low-grade dysplasia and patients with residual low molecular risk low-grade dysplasia. If prospective studies with large number of patients confirm my study findings, the future treatment of oral cancer patients with residual low-grade dysplasia may be drastically changed. This could have a significant impact on such patients, and hopefully, lead to an improvement in their dismal prognosis.

7.5   *Summary*

The results of this study, if confirmed by large prospective studies, will have important clinical implication in the management of patients with oral squamous cell carcinomas. On one hand, the study results confirm the value of the gold standard histology in judging the cancer risk of tumor surgical margins since an absence of the dysplasia correlated significantly with an absence of a high-risk molecular profile. Once there is dysplasia, the study results showed that the overall cancer risk of low-grade dysplasia taken from adjacent to cancer is markedly higher than primary lesions with similar histology; and that molecular markers could serve as powerful tool to identify high-risk, low-grade lesions from morphologically similar low-risk lesion, which the traditional gold standard histology is poor in differentiating. Salvage treatment of tumors with high-risk, low-grade dysplasia involving the surgical margins could prevent the development of the recurrent tumors.

8 References

Adamson R, Jones AS, Field JK. **Loss of heterozygosity studies on chromosome 17 in head and neck cancer using microsatellite markers.** *Oncogene* 1994 Jul; 9(7): 2077-82.

Ah-See KW, Cooke TG, Pickford IR, Soutar D, Balmain A. **An allelotype of squamous carcinoma of the head and neck using microsatellite markers.** *Cancer Res* 1994; 54: 1617-21.

Al-Rajhi N, Khafaga Y, El-Husseiny J, Saleem M, Mourad W, Al-Otieschan A, Al-Amro A. **Early stage carcinoma of oral tongue: prognostic factors for local control and survival.** *Oral Oncol* 2000 Nov; 36(6): 508-14.

Andre K, Schraub S, Mercier M, Bontemps P. **Role of alcohol and tobacco in the aetiology of head and neck cancer: a case-control study in the Doubs region of France.** *Eur J Cancer B Oral Oncol* 1995 Sep; 31B(5): 301-9.

BC Cancer Agency website.
http://www.bccancer.bc.ca/HPI/CancerManagementGuidelines/HeadnNeck/Dentistry/23505OralDentalManagementofPatientsReceivingRadiationTherapy.htm

Bedi GC, Westra WH, Gabrielson E, Koch W, Sidrandsky D. **Multiple head and neck tumors: evidence for a common clonal origin.** *Cancer Res* 1996; 56: 2484-7.

Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW. **Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer.** *J Natl Cancer Inst* 1993; 85: 1159-64.

Blot WJ, McLaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A, Fraumeni JF Jr. **Smoking and drinking in relation to oral and pharyngeal cancer.** *Cancer Res* 1988 Jun 1; 48(11): 3282-7.

Bockmuhl, U., Schwendel, A., Dietel, M., Petersen, I. **Distinct patterns of chromosomal alterations in high- and low-grade head and neck squamous cell carcinomas.** *Cancer Res* 1996; 56: 5325-9.

Brown JS, Kalavrezos N, D'Souza J, Lowe D, Magennis P, Woolgar JA. **Factors that influence the method of mandibular resection in the management of oral squamous cell carcinoma.** *Br J Oral Maxillofac Surg* 2002 Aug; 40(4): 275-84.

Brunneman KD, Scott D. **N-nitrosomorpholine and other volatile N-nitrosamines in snuff tobacco.** *Carcinogenesis* 1982; 3: 693-6.

Bundgaard T, Bentzen SM, Wildt J. **The prognostic effect of tobacco and alcohol consumption in intra-oral squamous cell carcinoma**. *Eur J Cancer B Oral Oncol* 1994 Sep; 30B(5): 323-8.

Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. **Human cytochrome P-450PA (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines**. *Proc Natl Acad Sci U S A* 1989; 86: 7696-700.

Califano J, Leong PL, Koch WM, Eisenberger CF, Sidransky D, Westra WH. **Second Esophageal Tumors in Patients with Head and Neck Squamous Cell Carcinoma: An Assessment of Clonal Relationships**. *Clinical Cancer Research* 1999; 5: 1862–1867.

Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, Sidransky D. **Genetic progression model for head and neck cancer: implications for field cancerization**. *Cancer Res* 1996 Jun 1; 56(11): 2488-92.

Califano J, Westra WH, Meininger G, Corio R, Koch WM, Sidransky D. **Genetic Progression and Clonal Relationship of Recurrent Premalignant Head and Neck Lesions**. *Clinical Cancer Research* 2000 Feb; 6: 347–352.

Croce CM, Sozzi G, Huebner K. **Role of FHIT in human cancer**. *J Clin Oncol* 1999; 17: 1618-24.

Crowe DL, Hacia JG, Hsieh CL, Sinha UK, Rice H. **Molecular pathology of head and neck cancer.** *Histol Histopathol* 2002; 17(3): 909-14.

Dawson CD, Chang KW, Solt DB. **MTS1 gene mutations in archival oral squamous cell carcinomas.** *J Oral Pathol Med* 1996; 25: 541-6.

Decker HJ, Weidt EJ, Brieger J. **The von Hippel-Lindau tumor suppressor gene. A rare and intriguing disease opening new insight into basic mechanisms of carcinogenesis.** *Cancer Genet Cytogenet* 1997; 93: 74-83.

De Vicente JC, Recio OR, Pendas SL, Lopez-Arranz JS. **Oral squamous cell carcinoma of the mandibular region: A survival study.** *Head Neck* 2001 Jul; 23(7): 536-43.

El-Naggar AK, Coombes MM, Batsakis JG, Hong WK, Goepfert H, Kagan J. **Localization of chromosome 8p regions involved in early tumorigenesis of oral and laryngeal squamous carcinoma.** *Oncogene* 1998; 16: 2983-7.

Emilion G, Langdon JD, Speight P, Partridge M. **Frequent gene deletions in potentially malignant oral lesions.** *Br J Cancer* 1996; 73: 809-13.

Fearon ER. **Human cancer syndromes: clues to the origin and nature of cancer.**

Science 1997; 278: 1043-50.

Feigelson HS, Ross RK, Yu MC, Coetzee GA, Reichardt JK, Henderson BE. **Genetic susceptibility to cancer from exogenous and endogenous exposures.** *J Cell Biochem Suppl* 1996; 25: 15-22.

Field JK, Kiaris H, Risk JM, Tsiriyotis C, Adamson R, Zoumpourlis V, Rowley H, Taylor K, Whittaker J, Howard P. **Allelotype of squamous cell carcinoma of the head and neck: fractional allele loss correlates with survival.** *Br J Cancer* 1995; 72: 1180-8.

Frederick MJ, Holton PR, Hudson M, Wang M, Clayman GL. **Expression of apoptosis-related genes in human head and neck squamous cell carcinomas undergoing p53-mediated programmed cell death.** *Clin Cancer Res* 1999 Feb; 5(2): 361-9.

Frederickson SM, Messing EM, Reznikoff CA, Swaminathan S. **Relationship between in vivo acetylator phenotypes and cytosolic N-acetyltransferase and O-acetyltransferase activities in human uroepithelial cells.** *Cancer Epidemiol Biomarkers Prev* 1994; 3: 25-32.

Funk GF, Karnell LH, Robinson RA, Zhen WK, Trask DK, Hoffman HT. **Presentation, treatment, and outcome of oral cavity cancer: a National Cancer Data Base report.** *Head Neck* 2002 Feb; 24(2): 165-80.

Gallo O, Chiarelli I, Boddi V, Bocciolini C, Bruschini L, Porfirio B. **Cumulative prognostic value of p53 mutations and bcl-2 protein expression in head-and-neck cancer treated by radiotherapy**. *Int J Cancer* 1999; 84: 573-9.

Harada H, Tanaka H, Shimada Y, Shinoda M, Imamura M, Ishizaki K. **Lymph node metastasis is associated with allelic loss on chromosome 13q12-13 in esophageal squamous cell carcinoma**. *Cancer Res* 1999; 59: 3724-9.

Hoffman D, Djordjevic MV, Fan, J. **Five leading US commercial brands of moist snuff in 1994: assessment of carcinogenic N-nitrosamines**. *J Natl Cancer Inst* 1995; 87: 1862-9.

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, et al. **Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck**. *N Engl J Med* 1990 Sep 20; 323(12): 795-801.

Hsu TC, Furlong C, Spitz MR. **Ethyl alcohol as a cocarcinogen with special reference to the aerodigestive tract: a cytogenetic study**. *Anticancer Res* 1991 May-Jun; 11(3): 1097-101.

Hsu TC, Shirley LR, Takanari H. **Cytogenetic assays for mitotic poisons: the diploid Chinese hamster cell system**. *Anticancer Res* 1983; 3 ; 155-9.

Ishwad CS, Shuster M, Bockmuhl U, Thakker N, Shah P, Toomes C, Dixon M, Ferrell RE, Gollin SM. **Frequent allelic loss and homozygous deletion in chromosome band 8p23 in oral cancer.** *Int J Cancer* 1999; 80: 25-31.

Jares P, Nadal A, Fernandez PL, Pinyol M, Hernandez L, Cazorla M, Hernandez S, Bea S, Cardesa A, Campo E. **Disregulation of p16MTS1/CDK4I protein and mRNA expression is associated with gene alterations in squamous-cell carcinoma of the larynx.** *Int J Cancer* 1999; 81: 705-11.

Khuri FR, Lippman SM, Spitz MR, Lotan R, Hong WK. **Molecular epidemiology and retinoid chemoprevention of head and neck cancer.** J Natl Cancer Inst 1997 Feb 5; 89(3): 199-211.

Knudson AG. **Hereditary cancer, oncogenes, and antioncogenes.** *Cancer Res* 1985; 45: 1437-43.

Koch WM, Lango M, Sewell D, Zahurak M, Sidransky D. **Head and neck cancer in nonsmokers: a distinct clinical and molecular entity.** *Laryngoscope* 1999 Oct; 109(10): 1544-51.

Kok K, Naylor SL, Buys CH. **Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes.** *Adv Cancer Res* 1997; 71: 27-92.

Lazar AD, Winter MR, Nogueira CP, Larson PS, Finnemore EM, Dolan RW, Fuleihan N, Chakravarti A, Zietman A, Rosenberg CL. **Loss of heterozygosity at 11q23 in squamous cell carcinoma of the head and neck is associated with recurrent disease.** *Clin Cancer Res* 1998; 4: 2787-93.

Leong PP, Rezai B, Koch WM, Reed A, Eisele D, Lee DJ, Sidransky D, Jen J, Westra WH. **Distinguishing second primary tumors from lung metastases in patients with head and neck squamous cell carcinoma.** *Journal of the National Cancer Institute* 1998; Vol. 90, No. 13: 972-7.

Li X, Lee NK, Ye YW, Waber PG, Schweitzer C, Cheng QC, Nisen PD. **Allelic loss at chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer.** *J Natl Cancer Inst* 1994; 86: 1524-9.

Liggett WH, Sewell DA, Rocco J, Ahrendt SA, Koch W, Sidransky D. **p16 and p16 beta are potent growth suppressors of head and neck squamous carcinoma cells in vitro.** *Cancer Res* 1996; 56: 4119-23.

Lippman SM, Hong WK. **Second malignant tumors in head and neck squamous cell carcinoma: the overshadowing threat for patients with early-stage disease.** *Int J Radiat Oncol Biol Phys* 1989 Sep; 17(3): 691-4.

Lumerman H, Freedman P, Kerpel S. **Oral epithelial dysplasia and the development of invasive squamous cell carcinoma.** *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1995; 79: 321-9.

Lydiatt WM, Davidson BJ, Schantz SP, Caruana S, Chaganti RS. **9p21 deletion correlates with recurrence in head and neck cancer.** *Head Neck* 1998 Mar; 20(2): 113-8.

Maestro R, Gasparotto D, Vukosavljevic T, Barzan L, Sulfaro S, Boiocchi M. **Three discrete regions of deletion at 3p in head and neck cancers.** *Cancer Res* 1993; 53: 5775-9.

Maestro R, Piccinin S, Doglioni C, Gasparotto D, Vukosavljevic T, Sulfaro S, Barzan L, Boiocchi M. **Chromosome 13q deletion mapping in head and neck squamous cell carcinomas: identification of two distinct regions of preferential loss.** *Cancer Res* 1996; 56: 1146-50.

Mao EJ, Schwartz SM, Daling JR, Beckmann AM. **Loss of heterozygosity at 5q21-22 (adenomatous polyposis coli gene region) in oral squamous cell carcinoma is common and correlated with advanced disease.** *J Oral Pathol Med* 1998 Aug; 27(7): 297-302.

Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D. **Microsatellite**

alterations as clonal markers for the detection of human cancer. *Proc Natl Acad Sci U S A* 1994 Oct 11; 91(21): 9871-5.

Mao L, Fan YH, Lotan R, Hong WK. **Frequent abnormalities of FHIT, a candidate tumor suppressor gene, in head and neck cancer cell lines.** *Cancer Res* 1996; 56: 5128-31.

Mao L. **Tumor suppressor genes: does FHIT fit?** *J Natl Cancer Inst* 1998; 90: 412-4.

Mao L. **Can molecular assessment improve classification of head and neck premalignancy?** *Clin Cancer Res* 2000 Feb; 6(2): 321-2.

Matsuda H, Konishi N, Hiasa Y, Hayashi I, Tsuzuki T, Tao M, Kitahori Y, Yoshioka N, Kirita T, Sugimura M. **Alterations of p16/CDKN2, p53 and ras genes in oral squamous cell carcinomas and premalignant lesions.** *J Oral Pathol Med* 1996; 25: 232-8.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D. **5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers.** *Nat Med* 1995; 1: 686-92.

Namazie A, Alavi S, Olopade OI, Pauletti G, Aghamohammadi N, Aghamohammadi M, Gornbein JA, Calcaterra TC, Slamon DJ, Wang MB, Srivatsan ES. **Cyclin D1**

amplification and p16(MTS1/CDK4I) deletion correlate with poor prognosis in head and neck tumors. *Laryngoscope* 2002 Mar; 112(3): 472-81.

Nawroz H, van der Riet P, Hruban RH, Koch W, Ruppert JM, Sidransky D. **Allelotype of head and neck squamous cell carcinoma.** *Cancer Res* 1994; 54: 1152-5.

O-charoenrat P, Rhys-Evans PH, Archer DJ, Eccles SA. O-charoenrat P, Rhys-Evans PH, Archer DJ, Eccles SA. **C-erbB receptors in squamous cell carcinomas of the head and neck: clinical significance and correlation with matrix metalloproteinases and vascular endothelial growth factors.** *Oral Oncol* 2002 Jan; 38(1): 73-80.

Ogawara K, Miyakawa A, Shiba M, Uzawa K, Watanabe T, Wang XL, Sato T, Kubosawa H, Kondo Y, Tanzawa H. **Allelic loss of chromosome 13q14.3 in human oral cancer: correlation with lymph node metastasis.** *Int J Cancer* 1998; 79: 312-7.

Ohta T, Fukuda M, Wanebo HJ, Jogo K, Yamaguchi S. **Behavior of the cell cycle-associated proteins in an unusual G0-arrestable cancer cell line.** *Exp Cell Res* 1996; 225: 85-92.

Papadimitrakopoulou VA, Hong WK. **Retinoids in head and neck chemoprevention.** *Proc Soc Exp Biol Med* 1997; 216: 283-90.

Papadimitrakopoulou V, Izzo J, Lippman SM, Lee JS, Fan YH, Clayman G, Ro JY, Hittelman WN, Lotan R, Hong WK, Mao L. **Frequent inactivation of p16INK4a in**

oral premalignant lesions. *Oncogene* 1997; 14: 1799-803.

Partridge M, Emilion G, Langdon JD. **LOH at 3p correlates with a poor survival in oral squamous cell carcinoma.** *Br J Cancer* 1996; 73: 366-71.

Partridge M, Emilion G, Pateromichelakis S, A'Hern R, Phillips E, Langdon J. **Allelic imbalance at chromosomal loci implicated in the pathogenesis of oral precancer, cumulative loss and its relationship with progression to cancer.** *Oral Oncol* 1998; 34: 77-83.

Partridge M, Emilion G, Pateromichelakis S, Phillips E, Langdon J. **Location of candidate tumour suppressor gene loci at chromosomes 3p, 8p and 9p for oral squamous cell carcinomas.** *Int J Cancer* 1999; 83: 318-25.

Partridge M, Pateromichelakis S, Phillips E, Emilion G, Langdon J. **Profiling clonality and progression in multiple premalignant and malignant oral lesions identifies a subgroup of cases with a distinct presentation of squamous cell carcinoma.** *Clin Cancer Res* 2001 Jul; 7(7): 1860-6.

Partridge M, Kiguwa S., Emilion G, Pateromichelakis S, A'Hern R, Langdon JD. **New insights into p53 protein stabilization in oral squamous cell carcinoma.** *Oral Oncol* 1999b; 35: 45-55.

Paterson IC, Eveson JW, Prime SS. **Molecular changes in oral cancer may reflect aetiology and ethnic origin**. *Eur J Cancer B Oral Oncol* 1996 May; 32B(3): 150-3.

Paterson IC, Matthews JB, Huntley S, Robinson CM, Fahey M, Parkinson EK, Prime SS. **Decreased expression of TGF-beta cell surface receptors during progression of human oral squamous cell carcinoma**. *J Pathol* 2001 Apr; 193(4): 458-67.

Pavelic K, Krizanac S, Cacev T, Hadzija MP, Radosevic S, Crnic I, Levanat S, Kapitanovic S. **Aberration of FHIT gene is associated with increased tumor proliferation and decreased apoptosis-clinical evidence in lung and head and neck carcinomas**. *Mol Med* 2001 Jul; 7(7): 442-53.

Pennisi E. **New gene forges link between fragile site and many cancers**. *Science* 1996; 272: 649.

Pershouse MA, El-Naggar AK, Hurr K, Lin H, Yung WK, Steck PA. **Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma**. *Oncogene* 1997; 14: 369-73.

Poh CF, Zhang L, Lam WL, Zhang X, An D, Chau C, Priddy R, Epstein J, Rosin MP. **A high frequency of allelic loss in oral verrucous lesions may explain malignant risk**. *Lab Invest* 2001 Apr; 81(4): 629-34.

Rawnsley JD, Srivatsan ES, Chakrabarti R, Billings KR, Wang MB. **Deletion analysis of the p16/CDKN2 gene in head and neck squamous cell carcinoma using quantitative polymerase chain reaction method**. *Arch Otolaryngol Head Neck Surg* 1997; 123: 863-7.

Raybaud-Diogene H, Tetu B, Morency R, Fortin A, Monteil RA. **p53 overexpression in head and neck squamous cell carcinoma: review of the literature**. *Eur J Cancer B Oral Oncol* 1996 May; 32B(3): 143-9.

Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, Sewell D, Nawroz H, Bartek J, Sidransky D. **High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma**. *Cancer Res* 1996; 56: 3630-3.

Roh HJ, Shin DM, Lee JS, Ro JY, Tainsky MA, Hong WK, Hittelman WN. **Visualization of the timing of gene amplification during multistep head and neck tumorigenesis**. *Cancer Res* 2000 Nov 15; 60(22): 6496-502.

Rosin MP, Cheng X, Poh C, Lam WL, Huang Y, Lovas J, Berean K, Epstein JB, Priddy R, Le ND, Zhang L. **Use of Allelic Loss to Predict Malignant Risk for Low-grade Oral Epithelial Dysplasia**. *Clinical Cancer Research* 2000; Vol. 6: 357–362.

Rosin MP, Lam WL, Poh C, Le ND, Li RJ, Zeng T, Priddy R, Zhang L. **3p14 and 9p21 loss is a simple tool for predicting second oral malignancy at previously treated oral cancer sites**. *Cancer Res* 2002 Nov 15; 62(22): 6447-50.

Roz L, Wu CL, Porter S, Scully C, Speight P, Read A, Sloan P, Thakker N. **Allelic imbalance on chromosome 3p in oral dysplastic lesions: an early event in oral carcinogenesis**. *Cancer Res* 1996; 56: 1228-31.

Saranath D, Bhoite LT, Deo MG. **Molecular lesions in human oral cancer: the Indian scene**. *Eur J Cancer B Oral Oncol* 1993 Apr; 29B(2): 107-12.

Sartor M, Steingrimsdottir H, Elamin F, Gaken J, Warnakulasuriya S, Partridge M, Thakker N, Johnson NW, Tavassoli M. **Role of p16/MTS1, cyclin D1 and RB in primary oral cancer and oral cancer cell lines**. *Br J Cancer* 1999; 80: 79-86.

Schantz SP, Hsu TC. **Mutagen-induced chromosome fragility within peripheral blood lymphocytes of head and neck cancer patients**. *Head Neck* 1989 Jul-Aug; 11(4): 337-42.

Schepman KP, van der Meij EH, Smeele LE, van der Waal I. **Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands**. *Oral Oncol* 1998 Jul; 34(4): 270-5.

Scholes AG, Woolgar JA, Boyle MA, Brown JS, Vaughan ED, Hart CA, Jones AS, Field JK. **Synchronous oral carcinomas: independent or common clonal origin?** *Cancer Res* 1998 May 1; 58(9): 2003-6.

Scholnick SB, Haughey BH, Sunwoo JB, el-Mofty SK, Baty JD, Piccirillo JF, Zequeira MR. **Chromosome 8 allelic loss and the outcome of patients with squamous cell carcinoma of the supraglottic larynx.** *J Natl Cancer Inst* 1996; 88: 1676-82.

Scully C. **Oral precancer: preventive and medical approaches to management.** *Oral Oncol, Eur J Cancer* 1995; 31: 16-26.

Shin DM, Charuruks N, Lippman SM, Lee JJ, Ro JY, Hong WK, and Hittelman WN. **p53 Protein Accumulation and Genomic Instability in Head and Neck Multistep Tumorigenesis.** *Cancer Epidemiology, Biomarkers & Prevention* 2001 June; Vol. 10: 603–609.

Shin DM, Lee JS, Lippman SM, Lee JJ, Tu ZN, Choi G, Heyne K, Shin HJ, Ro JY, Goepfert H, Hong WK, Hittelman WN. **p53 expressions: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma.** *J Natl Cancer Inst* 1996 Apr 17; 88(8): 519-29.

Sidransky D, Frost P, Von Eschenbach A, Oyasu R, Preisinger AC, Vogelstein B.

Clonal origin bladder cancer. *N Engl J Med* 1992a Mar 12; 326(11): 737-40.

Sidransky D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein

B. **Clonal expansion of p53 mutant cells is associated with brain tumour**

progression. *Nature* 1992b Feb 27; 355(6363): 846-7.

Silverman S, Gorsky M, Lozada F. **Oral leukoplakia and malignant transformation.**

A follow-up study of 257 patients. *Cancer* 1984; 53: 563-8.

Slaughter DP, Southwick HW, Smejkal W. **"Field cancerization" in oral stratified**

squamous epithelium. *Cancer* 1953; 6: 963-8.

Sozzi G, Veronese ML, Negrini M, Baffa R, Cotticelli MG, Inoue H, Tornielli S, Pilotti

S, De Gregorio L, Pastorino U, Pierotti MA, Ohta M, Huebner K, Croce CM. **The FHIT**

gene 3p14.2 is abnormal in lung cancer. *Cell* 1996; 85: 17-26.

Spitz MR, Bondy ML. **Genetic susceptibility to cancer.** *Cancer* 1993; 72: 991-5.

Spitz MR, Newell GR. **Descriptive epidemiology of squamous cell carcinoma of the**

upper aerodigestive tract. *Cancer Bull* 1987; 39: 79-81.

Squire JA, Bayani J, Luk C, Unwin L, Tokunaga J, MacMillan C, Irish J, Brown D,

Gullane P, Kamel-Reid S. **Molecular cytogenetic analysis of head and neck squamous cell carcinoma: By comparative genomic hybridization, spectral karyotyping, and expression array analysis.** *Head Neck* 2002 Sep; 24(9): 874-87.

Stafford ND, Ashman J.N.E., MacDonald AW, Ell SR, Monson J.R.T., Greenman J. **Genetic Analysis of Head and Neck Squamous Cell Carcinoma and Surrounding Mucosa.** *Arch Otolaryngol Head Neck Surg.* 1999; 125: 1341-1348.

Stott F J, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. **The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2.** *EMBO J.* 1998; 17: 5001-5014.

Sukosd F, Kuroda N, Beothe T, Kaur AP, Kovacs G. **Deletion of chromosome 3p14.2-p25 involving the VHL and FHIT genes in conventional renal cell carcinoma.** *Cancer Res* 2003 Jan 15; 63(2): 455-7.

Tabor MP, Brakenhoff RH, Ruijter-Schippers HJ, van der Wal JE, Snow GB, Leemans CR., and Braakhuis BJ. **Multiple Head and Neck Tumors Frequently Originate from a Single Preneoplastic Lesion.** *American Journal of Pathology* 2002 Sep; Vol. 161, No. 3: 1051-1060.

Tabor MP, Brakenhoff RH, van Houten V.M.M., Kummer JA, Snel M.H.J., J. F. Snijders P.J.F., Snow GB, Leemans CR, and Braakhuis BJ. **Persistence of Genetically Altered Fields in Head and Neck Cancer Patients: Biological and Clinical Implications.** *Clinical Cancer Research* 2001 June; Vol. 7: 1523–1532.

Takes RP, Baatenburg de Jong RJ, Wijffels K, Schuuring E, Litvinov SV, Hermans J, van Krieken JH. **Expression of genetic markers in lymph node metastases compared with their primary tumours in head and neck cancer.** *Pathol* 2001 Jul; 194(3): 298-302.

Tanimoto K, Hayashi S, Tsuchiya E, Tokuchi Y, Kobayashi Y, Yoshiga K, Okui T, Kobayashi M, Ichikawa T. **Abnormalities of the FHIT gene in human oral carcinogenesis.** *Br J Cancer* 2000 Feb; 82(4):838-43.

Todd R, McBride J, Tsuji T, Donoff RB, Nagai M, Chou MY, Chiang T, Wong DT. **Deleted in oral cancer-1 (doc-1), a novel oral tumor suppressor gene.** *FASEB J* 1995 Oct; 9(13): 1362-70.

U.S. Department of Health and Health Service. (1982). **The health consequences of smoking: cancer. A report of the Surgeon General.** US Department of Health and Human Services, Public Health Service.: Rockville, MD.

U.S. Department of Health, Education, and Welfare. (1973). **Pipe and cigar smoking. In**

the health consequences of smoking. US Department of Health, Education, and Welfare, Public Health Service.: Washington, DC.

U.S. Department of Health, Education, and Welfare. (1979). **Smoking and health. A report of the Surgeon General**. US Department of Health, Education, and Welfare, Public Health Service, Office of Smoking and Health.: Washington, DC.

Uzawa K, Yoshida H, Suzuki H, Tanzawa H, Shimazaki J, Seino S, Sato K. **Abnormalities of the adenomatous polyposis coli gene in human oral squamous-cell carcinoma**. *Int J Cancer* 1994; 58: 814-7.

Uzawa N, Yoshida MA, Hosoe S, Oshimura M, Amagasa T, Ikeuchi T. **Functional evidence for involvement of multiple putative tumor suppressor genes on the short arm of chromosome 3 in human oral squamous cell carcinogenesis**. *Cancer Genet Cytogenet* 1998; 107: 125-31.

Uzawa N, Akanuma D, Negishi A, Iwaki H, Uzawa Y, Amagasa T, Yoshida MA. **Homozygous deletions on the short arm of chromosome 3 in human oral squamous cell carcinomas**. *Oral Oncol* 2001 Jun; 37(4):351-6.

van den Berg A, Buys CH. **Involvement of multiple loci on chromosome 3 in renal cell cancer development**. *Genes Chromosomes Cancer* 1997; 19: 59-76.

van der Tol IGH, de Visscher JGAM, Jovanovic A, van der Waal I. **Risk of second primary cancer following treatment of squamous cell carcinoma of the lower lip**. *Oral Oncology* 1999; 35: 571-574.

van der Waal I, Schepman KP, van der Meij EH, Smeele LE. **Oral leukoplakia: a clinicopathological review**. *Oral Oncol* 1997 Sep; 33(5): 291-301.

van Heerden WF, Swart TJ, Robson B, Smith TL, Engelbrecht S, van Heerden MB, van Rensburg EJ, Huebner K. **FHIT RNA and protein expression in oral squamous cell carcinomas.** *Anticancer Res* 2001 Jul-Aug; 21(4A): 2425-8.

Van Houten VM, Tabor MP, Van Den Brekel MW, Alain Kummer J, Denkers F, Dijkstra J, Leemans R, Van Der Waal I, Snow GB, Brakenhoff RH. **Mutated p53 as a molecular marker for the diagnosis of head and neck cancer.** *J Pathol* 2002 Dec; 198(4): 476-486.

Venugopalan M, Wood TF, Wilczynski SP, Sen S, Peters J, Ma GC, Evans GA, Srivatsan ES. **Loss of heterozygosity in squamous cell carcinomas of the head and neck defines a tumor suppressor gene region on 11q13.** *Cancer Genet Cytogenet* 1998; 104: 124-32.

Vikram B, Chadha M, Malamud S, Yu L. **Patterns of failure in carcinoma of the upper esophagus after alternating chemoradiotherapy.** *Am J Surg* 1994 Nov; 168(5):

423-4.

W.H.O. **Definition of leukoplakia and related lesions: an aid to studies on oral precancer.** *Oral Surg Oral Med Oral Pathol* 1978; 46: 518-39.

Waber P, Dlugosz S, Cheng QC, Truelson J, Nisen PD. **Genetic alterations of chromosome band 9p21-22 in head and neck cancer are not restricted to p16INK4a.** *Oncogene* 1997 Oct 2; 15(14): 1699-704.

Waber PG, Lee NK, Nisen PD. **Frequent allelic loss at chromosome arm 3p is distinct from genetic alterations of the Von-Hippel Lindau tumor suppressor gene in head and neck cancer.** *Oncogene* 1996; 12: 365-9.

Warren S, Gates O. **Multiple primary malignant tumors. A survey of the literature and a statistical study.** *Am J Cancer* 1932; 16: 358–1414.

Weber A, Bellmann U, Bootz F, Wittekind C, Tannapfel A. **INK4a-ARF alterations and p53 mutations in primary and consecutive squamous cell carcinoma of the head and neck.** *Virchows Arch* 2002 Aug; 441(2): 133-42.

Wilke CM, Hall BK, Hoge A, Paradee W, Smith DI, Glover TW. **FRA3B extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites.** *Hum Mol Genet* 1996; 5: 187-95.

Winn DM, Blot WJ, Shy CM. **Snuff dipping and oral cancer among women in the southern United States.** *N Engl J Med* 1981; 304: 745-9.

Wu CL, Sloan P, Read AP, Harris R, Thakker N. **Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma of the oral cavity.** *Cancer Res* 1994; 54: 6484-8.

Wu CL, Roz L, Sloan P, Read AP, Holland S, Porter S, Scully C, Speight PM, Thakker N. **Deletion mapping defines three discrete areas of allelic imbalance on chromosome arm 8p in oral and oropharyngeal squamous cell carcinomas.** *Genes Chromosomes Cancer* 1997; 20: 347-53.

Yoo GH, Xu HJ, Brennan JA, Westra W, Hruban RH, Koch W, Benedict WF, Sidransky D. **Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma.** *Cancer Res* 1994; 54: 4603-6.

Xu J, Gimenez-Conti IB, Cunningham JE, Collet AM, Luna MA, Lanfranchi HE, Spitz MR, Conti CJ. **Alterations of p53, cyclin D1, Rb, and H-ras in human oral carcinomas related to tobacco use.** *Cancer* 1998 Jul 15; 83(2): 204-12.

Zhang L, Michelsen C, Cheng X, Zeng T, Priddy R, Rosin MP. **Molecular analysis of oral lichen planus. A premalignant lesion?** *Am J Pathol* 1997; 151: 323-7.

Zhang L, Epstein J, Band P, Berean K, Hay J, Cheng X, Rosin MP. **Local tumor recurrence or emergence of a new primary lesion? A molecular analysis.** *J Oral Pathol Med* 1999 Sep; 28(8): 381-4.

Zhang L, Cheng X, Li Y, Poh C, Zeng T, Priddy R, Lovas J, Freedman P, Daley T, Rosin MP. **High frequency of allelic loss in dysplastic lichenoid lesions.** *Lab Invest* 2000 Feb; 80(2): 233-7.

Zhang L, Cheung KJ Jr, Lam WL, Cheng X, Poh C, Priddy R, Epstein J, Le ND, Rosin MP. **Increased genetic damage in oral leukoplakia from high risk sites: potential impact on staging and clinical management.** *Cancer* 2001 Jun 1; 91(11): 2148-55.

Zhang L, Poh CF, Lam WL, Epstein JB, Cheng X, Zhang X, Priddy R, Lovas J, Le ND, Rosin MP. **Impact of localized treatment in reducing risk of progression of low-grade oral dysplasia: molecular evidence of incomplete resection.** *Oral Oncol* 2001 Sep; 37(6): 505-12.

88

Zhang L, Epstein JB, Poh CF, Berean K, Lam WL, Zhang X, Rosin MP. **Comparison of HPV infection, p53 mutation and allelic losses in post-transplant and non-posttransplant oral squamous cell carcinomas.** *J Oral Pathol Med* 2002 Mar; 31(3): 134-41.

9 Appendix A

9.1 Data for primary hyperplasia

Patient ID	Site	Risk	Age	Gender	Smoking	3pLOH	4qLOH	8pLOH	9pLOH	11qLOH	13qLOH	17pLOH
0024	tongue	H	64	F	N	R	R	NI	NI	R	R	R
0028	r buccal mucosa	L	51	F	N	R	R	R	R	R	R	R
0127	gum	L	42	M	S	NI	R	R	R	R	R	R
0140	hard palate	L	52	F	S	R	R	R	R	R	R	R
0173	edge l tong	H	79	M	S	R	R	L	R	R	R	R
0359	r cheek	L	63	M	S	R	R	R	R	R	R	R
0416	max tuberosity	L	57	M	S	L	R	R	R	R	R	R
0435	l lat tong	H	42	F	N	R	R	R	R	R	R	R
0596	r buccal mucosa	L	57	M	na	R	NI	R	R	R	R	R
0598	tongue	H	62	M	S	R	R	R	R	R	R	R
0146	gingiva/maxilla	L	16	M	N	R	R	R	L	R	R	R
0149	buccal sulcus of r cheek beside 27	L	20	M	N	R	R	R	R	R	NI	R
0150	ventral surface of tong	H	32	M	N	R	NI	R	R	R	R	R
0151	l buccal mucosa	L	76	M	S	R	R	R	R	R	R	R
0152	hard palate	L	38	F	S	R	R	R	R	R	R	R

0211	r buccal mucosa	L	70	M	S	R	R	R	R	R	R	R
0285	r lat tong	H	63	M	S	R	NI	N	R	N	N	N
0288	hard palate	L	69	M	N/A	R	R	NI	R	R	R	R
0599	l upper alveolus	L	83	F	NA	R	R	R	R	R	R	R
0506	buccal	L	33	M	S	R	R	R	R	R	R	R
0507	r buccal mucosa	L	27	M	S	R	NI	R	R	R	R	R
0508	l buccal mucosa	L	54	M	S	R	R	R	R	R	R	R
0509	r lat tong	H	64	F	S	R	R	R	R	R	R	R
0523	l mandibular retromolar pad	L	60	M	na	R	R	R	R	R	R	R
0483	gum	L	78	F	N	R	R	NI	R	R	R	R.
0569	midline of alveolus	L	74	F	N	R	R	NI	R	R	R	L
0011	17 region maxilla	L	37	M	N	R	R	R	R	R	R	R
0101	right tong	H	41	F	N	R	R	R	R	R	R	R
0102	48 & 31 - 41	L	24	F	N	R	NI	R	R	R	R	R
0103	interproximal gum	L	36	F	N	R	R	R	R	R	R	R

List of abbreviations:
For the sites: r = right, l = left, lat = lateral, ant = anterior, tong = tongue, FOM = floor of mouth.
For LOH results: L = loss of heterozygosity, R = retention, NI = non-informative, NA = data not available.

9.2 Data for hyperplasia adjacent to cancers

Patient ID	Site	Risk	Age	Gender	Smoking	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
0110	tong	H	39	F	S	R	R	R	R	R	R	R
0117	lateral border of tong	H	34	M	N	R	R	R	R	R	NI	R
0123	lat tong	H	63	M	S	R	R	R	R	R	R	R
0125	r gingiva	L	85	F	N	R	R	R	R	R	R	R
0161	r tong	H	46	M	NA	R	R	R	R	R	R	R
0162	l ventral tong	H	55	F	S	R	R	R	R	R	R	R
0174	ventral border of tong	H	36	M	S	R	R	NI	R	R	R	R
0196	tong	H	54	F	S	R	R	R	R	R	R	R
0197	l lat tong	H	42	M	S	R	NI	R	R	R	R	R
0212	l tong	H	58	M	NA	R	R	R	R	R	R	R
0220	l tong	H	35	M	N	L	NI	L	L	L	L	R
0228	dorsum of tong	H	35	M	N	R	R	R	R	R	R	R
0238	unknown	L	91	M	N	R	NA	NA	R	NA	NA	R
0376	r base of tong	H	62	M	S	NA	NA	NA	NA	NA	NA	NA
0043	lat tong	H	67	M	S	R	R	R	R	R	R	R

List of abbreviations:
For the sites: r = right, l = left, lat = lateral, ant = anterior, tong = tongue, FOM = floor of mouth.
For LOH results: L = loss of heterozygosity, R = retention, NI = non-informative, NA = data not available.

9.3   Data for primary low-grade dysplasia

Patient ID	Site	Risk	Age	Gender	Smoking	3pLOH	4qLOH	8pLOH	9pLOH	11qLOH	13qLOH	17pLOH
0187	l soft palate	L	41	F	S	R	R	R	L	R	R	L
0188	r FOM	H	68	F	S	R	R	R	L	R	R	R
0197	l lat tong	H	39	M	S	R	NI	R	L	R	R	R
0200	gingiva	L	49	F	N	L	R	R	L	R	R	L
0223	tong	H	29	M	S	R	R	R	L	R	R	R
0286	r buccal mucosa	L	65	M	na	L	R	L	R	R	L	L
0289	r hard palate	L	39	M	S	R	N	N	R	N	N	R
0290	post buc vestibule	L	59	N/A	na	R	N	N	R	N	N	R
0291	L upper lip	H	71	M	na	R	N	N	L	N	N	R
0292	upper l canine region	L	78	N/A	N	R	N	N	R	N	N	R
0293	tong	H	45	M	na	L	R	R	L	R	R	L
0295	l post hard palate	L	62	F	S	R	N	N	R	N	N	L
0296	gingiva	L	36	M	S	R	N	N	R	N	N	R
0297	1.4 &1.3	L	33	M	S	R	N	N	R	N	N	R
0300	r mandibular alveolar ridge	L	78	F	na	R	N	N	R	N	N	R

0316	r buccal mucosa	L	46	M	S	R	N	R	N	N	R	N	N	R
0347	L lower vestibule	L	71	F	na	R	N	R	N	N	L	N	N	R
0460	ant FOM	H	46	M	S	R	R	R	L	R	R	L	L	R
0464	tong	H	71	F	na	R	L	R	R	L	R	R	R	L
0567	FOM	H	60	F	N	R	R	R	R	R	L	R	R	R
0567	FOM	H	60	F	N	R	NI	R	R	NI	L	R	R	R
0569	frenum of tong	H	74	F	N									
0570	tong	H	56	F	na	R	NI	R	R	NI	R	R	R	R
0571	FOM	H	46	M	S	L	R	L	NI	R	R	NI	R	R
0575	r tong	H	74	F	na	L	R	L	R	R	NI	R	R	R
0576	tong	H	60	M	S	R	L	R	L	L	L	L	R	L
0577	l tong	H	51	M	S	NI	R	NI	R	R	R	R	NI	R
0578	tong	H	55	F	S	R	R	R	R	R	R	R	NI	R
0620	l lat tong	H	55	M	S	R	R	R	R	R	R	R	R	NI
0634	Soft palate	L	43	F	N	R	L	R	R	L	R	R	R	R
0675	l soft palate	L	45	f	S	R	R	R	R	R	R	R	N	NI
0680	r lat tong	H	55	M	na	R	R	R	R	R	NI	NI	N	L
0681	r ventral tong	H	71	F	na	R	R	R	R	R	L	NI	N	R
0682	r FOM	H	40	f	na	L	R	L	R	R	R	N	N	R
0683	r FOM	H	54	M	S	R	R	R	R	R	R	R	N	R
0288	hard palate	L	61	M	S	R	R	R	R	R	R	R	R	R,R
0301	labial vestibule	L	67	F	S	R	R	R	R	R	R	R	R	R

ID	Site		Age	Sex									
0364	l post cheek	L	60	M	S	NI	N	N	R	NI	R	R	R
0418	r FOM	H	83	M	N	R	R	L	R	L	R	R	L
0618	lingual	L	75	F	S	R	R	R	L	R	R	R	R
0630	ant vent tong	H	66	M	N	L	R	L	R	R	R	R	R
0634	Soft palate	L	43	F	N	R	R	L	R	R	R	R	R
0638	l vent tong	H	67	F	N	L	R	L	R	L	L	R	R
0386	r soft palate	L	65	M	S	R	R	R	NI	R	R	R	R
0688	r lat tong	H	62	F	S	R	R	R	L	L	R	R	R

List of abbreviations:

For the sites: r = right, l = left, lat = lateral, ant = anterior, tong = tongue, FOM = floor of mouth.

For LOH results: L = loss of heterozygosity, R = retention, NI = non-informative, NA = data not available.

9.4 Data for low-grade dysplasia adjacent to cancers

Patient ID	Site	Risk	Age	Gender	Smoking	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
0010	r lat tong	H	29	F	N	R	L	L	L	R	R	L
0010	r lat tong	H	29	F	N	R	R	R	L	L	R	L
0010	r lat tong	H	33	F	N	R	R	L	R	L	L	L
0010	r lat tong	H	43	F	N	R	R	R	R	R	R	R
0113	1.1 & 1.2	L	30	F	NA	R	R	R	L	R	NI	R
0114	gingiva	L	75	F	S	R	L	R	L	R	L	R
0117	lat border of tong	H	35	M	N	R	L	R	L	R		R
0117	lat border of tong	H	35	M	NA	R	R	R	L	R	NI	R
0161	r tong	H	47	M	NA	R	R	R	R	R	NI	R
0162	l tong	H	47	F	S	R	R	R	R	R	R	R
0173	l tong	H	75	M	S	L	R	R	L	L	R	R
0175	l border of tong	H	30	F	N	R	R	L	L	R	L	L
0196	border of tong	H	51	F	S	R	R	R	R	L	R	R
0197	l lat tong	H	41	M	S	R	NI	R	R	R	R	R
0197	l lat tong	H	39	M	S	R	NI	L	L	R	R	R
0199	r tong	H	89	F	NA	L	L	L	L	R	R	L
0199	r tong	H	89	F	NA	L	R	L	L	R	R	L

ID	Site												
0200	post lat mouth	H	48	F	N	L	R	R	L	R	R	L	
0200	post lat mouth	H	48	F	N	L	R	R	L	R	R	L	
0204	l lat tong	H	47	M	N	L	L	NI	L	L	L	L	
0211	R buccal mucosa	L	79	M	S	R	R	R	R	R	R	R	
0212	l tong	H	58	M	NA	L	L	R	L	L	L	L	
0212	l tong	H	58	M	NA	L	R	R	R	R	R	R	
0213	vent tong	H	69	F	NA	R	L	L	L	L	L	R	
0215	r lat tong	H	53	M	NA	R	R	R	R	R	R	L	
0215	r lat tong	H	53	M	NA	R	R	R	R	R	R	L	
0200	l tong	H	35	M	N	R	NI	L	L	L	L	R	
0228	dorsum of tong	H	35	M	N	L	L	L	R	L	R	R	
0271	l tong	H	70	F	S	L	R	R	L	L	L	L	
0002	gingiva	L	58	F	S	R	R	R	L	L	L	R	
0383	FOM	H	57	M	S	NA	NA	NA	R	NA	NA	L	
0059	mandibular ant alveolar ridge	L	63	M	S	R	R	R	R	R	R	R	
0061	r tong	H	31	M	S	L	L	R	L	L	L	L	
0065	r base of tong	H	61	F	S	R	R	R	R	R	R	R	
0079	l tong	H	62	F	NA	R	L	NI	R	R	NI	R	
0080	ant FOM	H	77	M	N	L	L	L	L	L	R	R	
0083	r lat tong	H	66	M	N	R	R	R	R	R	R	R	
0090	FOM	H	76	M	S	R	R	R	R	R	R	R	

List of abbreviations:
For the sites: r = right, l = left, lat = lateral, ant = anterior, tong = tongue, FOM = floor of mouth.
For LOH results: L = loss of heterozygosity, R = retention, NI = non-informative, NA = data not available.