MOLECULAR ASSESSMENT OF FORMER CANCER SITES PREDICTS SECOND ORAL MALIGNANCY

By

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ABSTRACT

Clinicopathological criteria currently used to identify lesions at risk for second oral malignancy (SOM) have severe limitations. This thesis investigated the value of loss of heterozygosity (LOH) as a risk-predictor for SOM. Eighty-nine patients with a history of oral cancer in longitudinal follow-up were used. Each patient had one sample (biopsy or brushing) analyzed for LOH using 19 markers on 7 chromosome arms. Within the follow-up period (mean 65 months), 28% developed SOMs. Brushing served as a valid DNA source for LOH analysis. An increased frequency of LOH observed at several loci was noted in samples from the SOM group compared with the non-SOM: 3p ($P = 0.003$), 4q ($P = 0.045$), 9p ($P < 0.0001$), 17p ($P = 0.001$), multiple LOH ($P < 0.0001$), and LOH at 3p &/or 9p ($P < 0.0001$). The latter pattern was associated with a 21.4-fold increase in SOM risk. In conclusion, LOH analysis could identify high-risk lesions for SOM using either biopsies or brushings.

EXECUTIVE SUMMARY

The 5-year survival rate for oral cancer remains dismal at around 50%, mainly as a result of a high-rate of second oral malignancy (SOM), including both local recurrences and second primary tumors. Current identification of SOM is based on clinicopathological risk factors. Oral premalignant lesions (OPLs) are identified using clinical risk parameters, then biopsied and a histological evaluation is made for the presence/degree of dysplasia. Frequently, however, high-risk OPLs are not biopsied because clinicians can not differentiate them from reactive lesions induced by treatment of the cancer or are reluctant to repeatedly biopsy such treated fragile sites. Even when biopsied, treatment-induced reactive changes that often resemble low-grade dysplasia may hamper histological assessment of malignant risk. These severe limitations frequently cause failure in identifying and managing high-risk OPLs before their progression into SOM, despite intensive follow-up. New tools are needed to monitor the cancer sites noninvasively, and markers are needed to enhance our ability to assess the cancer risk of OPLs.

Recent studies including those from this laboratory, have shown that loss of heterozygosity (LOH) patterns could predict the cancer risk of OPLs, and that exfoliative cells obtained non-invasively could serve as a DNA source for LOH analysis. However, these studies are very preliminary in nature, all retrospective in design, and only from primary OPLs. Prospective studies, particularly those from patients with a history of

oral cancer, are needed to validate the use of LOH as a tool to predict cancer risk of OPLs using both biopsy samples and exfoliative cells obtained non-invasively.

The objectives of this thesis were to validate the use of exfoliative cells as a DNA source for LOH analysis, and the risk predictive value of LOH for SOM development in a longitudinal study.

Eighty-nine patients with a prior history of oral cancer being followed prospectively in the Oral Dysplasia Clinic were used. One sample per patient was taken from the prior cancer site for LOH analysis using 19 microsatellite markers on chromosome arms: 3p, 4q, 8p, 9p, 11q, 13q and 17p. The one sample included either a biopsy (when available) or exfoliative cells taken by brushing (when biopsy was unavailable). In addition clinicopathological data were collected in collaboration with other members of the research team, and included demographic, habit, and index tumor information, clinicopathological features of post-treatment cancer site during follow-up and outcome.

As of November 11, 2004, the end-date for data acquisition for this thesis, the mean follow-up time for the 89 patients was 5.5 years (65 ± 38 months). Within this follow-up period, 28% (25/89) had developed a SOM at the former cancer site (SOM group). The average time for the development of SOM was 35 (± 30) months, which is significantly shorter than the follow-up time for the 64 patients who did not develop SOM (non-SOM group) (62 ± 38; $P = 0.0009$). Demographics, smoking habit and features of the primary oral cancer did not predict SOM. However, the uptake of

toluidine blue (TB) and the presence of OPLs at the former cancer site were significantly more often apparent in the SOM group (TB: 45% *vs.* 12%; presence of OPLs: 92% *vs.* 48%).

Molecularly, the results showed that brushings could serve as a DNA source for LOH analysis. Of the 47 brushings, 49% had LOH on at least 1 region, 43% on 3p, 29% on 4q, 7% on 8p, 46% on 9p, 29% on 11q, 14% on 13q, and 18% on 17p. The percentage of patients with 3p and/or 9p LOH was significantly elevated in SOM group (56% *vs.* 10%, P = 0.0074).

A significantly higher frequency of LOH was noted in the SOM group: 3p (48% *vs.* 16% in non-SOM; $P = 0.0026$), 4q (32% *vs.* 11%; $P = 0.045$), 9p (72% *vs.* 22%; $P < 0.0001$) and 17p (61% *vs.* 11%, $P = 0.0011$). SOM lesions also showed significantly increased high-risk LOH patterns: multiple LOH (72% vs. 20% in non-SOM; $P < 0.0001$); LOH at 3p and/or 9p plus others chromosomes (64% vs. 19%; $P < 0.0001$); and LOH at 3p &/or 9p (84% vs. 31%; $P < 0.0001$). Survival analysis showed a 21.4-fold increase in risk of developing SOM for patients with 3p and/or 9p loss ($P < 0.0001$) with this risk showing little change when the additional loss at any of the other arms was considered. The latter was associated with a 21.8-fold increase in SOM risk.

In conclusion, the study results from this prospective study confirmed that LOH at 3p and/or 9p could identify high-risk OPLs for SOM development using either biopsies or brushings.

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ABBREVIATIONS

BCCA:	British Columbia Cancer Agency
CIS:	Carcinoma *in situ*
DNA:	Deoxyribonucleic acid
H&E:	Hematoxylin and eosin
LOH:	Loss of heterozygosity
OBS	Oral Biopsy Service
OCPL	Oral cancer prevention longitudinal study
OPL:	Oral premalignant lesion
PC:	Phenol-chloroform
PCR:	Polymerase chain reaction
PEP:	Primer-extension preamplification
RFLP:	Restriction fragment length polymorphism
SCC:	Squamous cell carcinoma
SDS:	Sodium dodecyl sulfate
SOM:	Second oral malignancy
SPT	Second primary tumor
TSG:	Tumor suppressor gene
SFU:	Simon Fraser University
STR:	Short tandem repeat
VC:	Verrucous carcinoma
WHO:	World Health Organization

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I. INTRODUCTION

I.1. Oral cancer

Oral squamous cell carcinoma (SCC) is the sixth most common malignancy worldwide with a global annual incidence exceeding 400,000 cases (WHO, 2004). Three quarters of these cancers are from the developing world, including about 65,000 cases from India (Bibhu *et al.,* 2002). For India, this represents up to 40% of cancers (Saranath *et al.,* 1993). In contrast, the disease is less common in the Western World, representing approximately 3% of all new cancers that occur annually (Harras *et al.,* 1996, Greenlee *et al.,* 2001). In Canada, about 3,100 new cases of oral cancer arise every year, and 1,050 of these result in death (National Cancer Institute of Canada 2004).

Oral SCC are believed to develop over decades, originating from oral premalignant lesions (OPLs) that show a histological progression from hyperplasia through increasing degrees of dysplasia, to carcinoma *in situ (CIS)* and finally invasive SCC. Once invasive cancer forms, prognosis is poor, with 5-year survival rates of about 40-50% in the Western world and even lower in India (20-43%) (Rao and Krishnamurthy, 1998; Greenlee *et al.*, 2001). In fact, the prognosis for oral cancer remains one of the worst among the major cancer types. Despite improvement of surgical techniques and adjuvant therapies, the survival rate for this disease has not improved in the last several decades. This is largely due to a high-rate of local regional recurrence as well as

development of second primary cancer (Khuri *et al.,* 1997; Holland, 1997; Lippman and Hong, 1989; Vikram *et a.,* 1994).

One key to improving the dismal mortality and morbidity rates of oral SCC is early diagnosis and management of oral premalignant lesions (OPLs). This is especially important for individuals with an oral cancer history, where the development of OPLs and their progression to recurrent tumors or second primary tumors is frequent. This thesis deals with the validation of a molecular approach that may facilitate the early diagnosis of high-risk oral premalignant lesions in patients with a history of oral cancer.

I.2. Anatomy and histology of normal oral mucosa

The oral cavity is bounded anteriorly and laterally by the teeth, the lower and upper gingiva, and the buccal mucosa, inferiorly by the floor of the mouth and tongue, superiorly by the hard and soft palates, and posteriorly by the soft palate and anterior and posterior pillars of the fauces (retromolar trigone). In the International Classification of Diseases for Oncology (ICD-O) coding system, cancer in the oral cavity includes codes from C00 to C06 (Pindborg *et al.,* WHO, 1997).

The oral cavity is lined by the oral mucosa, which consists of overlying epithelium and underlying lamina propria. The latter contains blood and lymphatic vessels, small

nerves, fibroblasts, collagen, elastic fibers, and other extracellular matrix components. It functions to nourish and support the epithelial layer.

The overlying epithelium of oral mucosa is stratified squamous epithelium. The stratified squamous epithelial cells are composed of basal and prickle cells. The one-layered cuboid-shaped basal cells separate the overlying epithelium from the underlying connective tissue. They are the only cells that have the capacity to divide. When a basal cell divides, it may give rise to new basal cells or differentiate to form the larger polyhedral-shaped prickle cells. As the prickle cells mature, they push towards the surface, becoming long and flat and lying parallel to the surface, and are eventually desquamated. The cells are in intimate contact with each other.
A thin layer, the so-called basement membrane, separates the epithelium and lamina propria. Basement membranes are thin layers of a specialized extracellular matrix that form the supporting structure on which epithelial cells grow. This structure provides a mechanical support for the above epithelial tissue and also influences cellular behavior (Erickson and Couchman, 2000; Paulsson, 1992). Basement membranes consist of two layers: the basal lamina, produced by epithelial cells, and the lamina reticularis, produced by connective tissue cells. The basal lamina controls the orientation, intracellular organization (stratification), and attachment and migration of basal epithelial cells (Fine, 1991).

The lining epithelium of oral mucosa is usually non-keratinized except for the mucosa lining of the attached gingiva, hard palate, dorsal surface of the tongue, and lips. The

keratinization is related to their function as mucosa at these sites, particularly the masticatory mucosa (of the hard palate and gingiva), which is often exposed to mechanical friction. Over 90% of the oral cancers arise from the stratified squamous epithelium that lines the oral cavity.

I.3. Oral premalignant lesions

A precancerous lesion is defined as morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart (WHO, 1978). As implied in the definition of the premalignancy, these lesions have an increased likelihood of cancer development; however, the majority of premalignant lesions do not become cancerous. Currently clinicopathological features are used to predict which premalignant lesions will become malignant. However, these features are far from adequate as risk predictors, as demonstrated by the wide variation of reported cancer transformation rates for OPLs identified clinically, with values from 0.13% to over 50% of cases in individual studies (Silverman et al., 1984; Rosati, 1994; Bouquot et al., 1994; Roz et al., 1996; Papadimitrakopoulou et al., 1997; Lee et al., 2000; Soukos, 2001; Amagasa et al., 1985). In the following sections the clinicopathological risk factors and their limitations are discussed.

I.3.1. Clinical risk factors used by clinicians to determine when and where to biopsy

The current diagnostic procedure for OPLs is first, identification of OPLs by clinicians, then an estimation of the risk of the OPLs. If the risk is determined to be reasonably high, a biopsy will be taken for histological examination. The clinical identification and risk prediction mainly involve four factors: clinical appearance, site and size of OPLs, and history of head and neck cancer.

The site of leukoplakia affects the risk of these lesions. Leukoplakias occur throughout the oral cavity, with those in the buccal and mandibular sites being the most common. Leukoplakias from the floor of the mouth, ventrolateral surface of the tongue and soft palate hold an increased cancer risk. Hence, these regions are called high-risk areas whereas the other oral sites are called low-risk areas (Schell and Schonberger, 1987; Mashberg and Meyers, 1976). However, some of the lesions located at the low-risk sites do progress into cancer (high-risk); whereas many of lesions at high risk sites do not progress into cancer. Site alone as a risk predictor is not sufficient.

The size of OPLs is another risk factor. The bigger the premalignant lesion a patient has, the higher the cancer risk. The concept of size here refers to the combination of all leukoplakias in the oral cavity if there is more than one leukoplakia. The cutoff size for different risks remains speculative. It is not clear what the risk is for different sizes of OPLs (Schepman and van der Waal, 1995; van der Waal et al., 2000; Axell et al., 1996; Pindborg et al., 1968). Most oral lesions fall into the category of less than 2 cm and have a low cancer risk.

Clinical appearance and history of oral cancer are two very important clinical risk factors and these are reviewed in the following sections.

I.3.1.1. Clinical appearance of OPLs

OPLs could either be clinically visible or clinically not visible/obvious. Lesions that are subclinical are only detectable pathologically and molecularly and are often missed since the required biopsies for identifying such lesions are not taken. Our current inability to detect a large proportion of early high-risk lesions clinically is supported by the fact that about half or more of oral SCC in Western countries appear to arise "out of the blue" and do not appear to be associated with visible OPLs (Schepman *et al.*, 1999).

When the OPLs are visible, most are present as leukoplakia and the remaining few appearing as erythroplakia. The term leukoplakia is used to designate a clinical "white patch" that occurs on mucous membranes such as the mucosa of the oropharynx, larynx, esophagus, and genital tract. In actual fact, leukoplakia may sometimes appear yellow to light brown, especially in smokers. The World Health Organization (1978) defines leukoplakia in the oral cavity as a white patch or plaque of oral mucosa that cannot be characterized clinically or pathologically as any other diagnosable disease and can not be removed by scraping. Usually a definitive diagnosis of oral leukoplakia is made as a result of the identification, and if possible, elimination of suspected etiological factors (Axell *et al.*, 1996). The WHO defines erythroplakia as a fiery red patch that cannot be characterized clinically or pathologically as any other definable lesion (Pindborg *et al.*,

WHO 1997). Some erythroplakias are smooth and some are granular, velvety, or nodular. Often there is a well-defined margin. The soft palate, ventral surface of the tongue, and floor of the mouth are the most likely sites to be involved. Since most visible OPLs present as leukoplakia, the terms OPL and leukoplakia are frequently used interchangeably.

The clinical appearance of OPLs affects the malignant risk of lesions. According to their clinical appearance, leukoplakias/OPLs may be classified as either homogeneous or non-homogeneous. Homogeneous leukoplakias are those lesions showing a consistent color and texture. These lesions are predominantly white, flat, thin, and rather smooth surfaced, although shallow cracks and slightly wrinkled surface with consistent texture are accepted (Pindborg *et al.,* WHO 1997). In general, homogeneous leukoplakias have low risk for malignant transformation (Axell *et al.,* 1996). In contrast, non-homogeneous leukoplakias, which account for about 10% of all leukoplakias, are those lesions with variations in either color (white-red) and/or in topographic appearance (exophytic, papillary, verrucous, nodular). In general, they demonstrate an increased risk of malignant transformation when compared to the homogeneous types (Axell *et al.,* 1996; Pindborg *et al.,* WHO 1997). Erythroplakia, one type of nonhomogeneous leukoplakia has very high cancer risk. In fact, most of these are either high-risk precancerous lesions or already cancer (Bouguot and Ephros, 1995; Mashberg, 1977; Waldron and Shafer, 1975).

While the clinical appearance of OPLs has cancer risk prediction value, particularly in those cases of obvious nodular/verrucous leukoplakia or erythroplakia, clinical appearance is frequently inadequate in judging the cancer risk. Even the most innocuous homogeneous leukoplakia could turn out to be carcinoma *in situ (CIS).* More importantly, many reactive lesions with no precancerous predisposition could clinically mimic either leukoplakia (e.g., reactive hyperplasia and frictional hyperkeratosis) or erythroplakia (e.g., inflammation). These reactive lesions are very common and far outnumber leukoplakias. They are frequently misdiagnosed as leukoplakia. Likewise many leukoplakias are misdiagnosed as reactive lesions.

I.3.1.2. History of oral SCC and risk of second oral malignancy (SOM)

Second oral malignancies (SOMs) include both tumor recurrence and second primary tumors (SPTs). Local tumor recurrence is defined as the occurrence of another oral carcinoma within 3 years of the initial cancer and at a distance that is less than 2cm away from the primary carcinoma (Braakhuis *et al.,* 2002). To define second primary tumor (SPT), most investigators currently use the criteria of Warren and Gates that were published in 1932 which involves the presence of histologically malignant tumors for both the primary and SPT, with the second tumor being either topographically distinct from the first tumor or chronologically distinct from the first tumor (occurring 3 years after the first tumor). The probability of one being a metastasis of the other should be excluded.

OPLs in patients with a history of oral cancer are known to have higher cancer risk than similar appearing OPLs in patients without a history of oral cancer (see Table I-1 and Table I-2 for rate of recurrence and second primary tumor formation). OPLs in patients with a history of oral cancer can either become a recurrent tumor (i.e., occurring at the site of the previous oral SCC within 3 years of primary SCC treatment) or a second primary oral SCC (i.e., occurring at the primary cancer site but beyond the 3 year time limit or occurring at a distinctively different oral site). The identification of OPLs before they have a chance to progress to SOM could significantly decrease the morbidity and mortality of oral cancer.

However, the above-mentioned problem in the clinical diagnosis of OPLs in non-cancer patients is also present in patients with a history of cancer. In other words, OPLs may either not be clinically apparent, or if visible, they could be confused with non-premalignant reactive lesions, namely treatment-induced reactive changes which often resemble low-grade dysplasia and may hamper histological assessment of malignant risk (i.e. case showed in Figure 1: A & B). Furthermore, sites of previous oral cancer are generally very fragile and notorious for high occurrence of reactive lesions because of the aggressive treatment from surgery or radiation. Generally clinicians are reluctant to repeatedly biopsy these fragile sites. Consequently, even when patients with a history of oral cancer are vigorously followed up, frequently the OPLs are not treated and identified until SOMs are formed. Techniques that could identify the high-risk OPLs in these high-risk patients are highly desired.

Table I-1. Recurrence rate of oral and HNSCC

Study	Sample Size	% Recurrence	Duration of Follow-Up (Months)
von Dersten *et al.*, 1995	155	37%	-
Charuruks *et al.*, 1996	46	47%	2-55
Shin *et al.*, 1996	66	50%	-
Ball *et al.*, 1997	24	42%	24
Dhooge *et al.*, 1998	127	31%	30 (1-77)
Sardi *et al.*, 2000	25	32%	22 (15-31)

Table I-2. Incidence of secondary primary malignancy for oral and HNSCC

Study	Sample Size	% Second Primary	Duration of Follow Up (Months)
Shikhani *et al.*, 1986	1961	9.7%	-
Charuruks *et al.*, 1996	46	22%	6-76
Shin *et al.*, 1996	66	33%	-
Dhooge *et al.*, 1998	127	13.5%	30 (1-77)
Rafferty *et al.*, 2001	425	8.2%	-

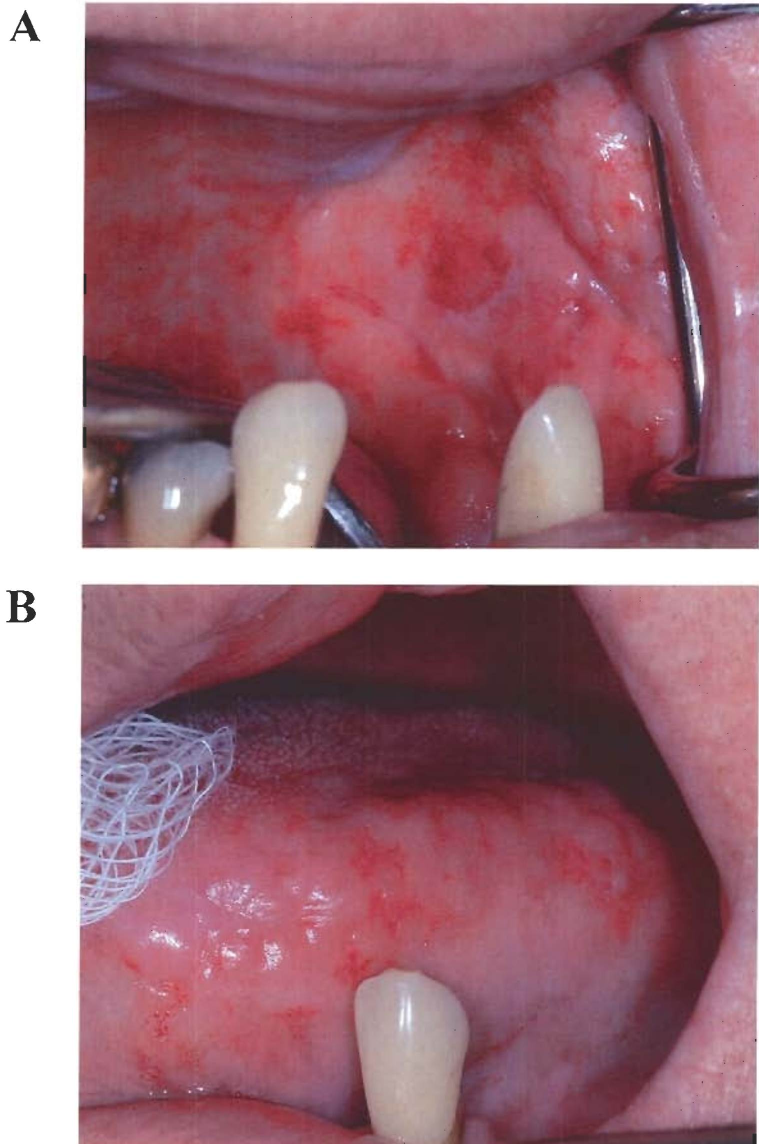


Figure 1. Non-premalignant reactive lesions confusing the identification of OPLs
This figure shows the clinical appearance of lesions in the oral cavity of a 64-year-old male smoker who had a history of a stage T2N0 cancer on the left mandible 1 year previously and had been treated by surgery and adjuvant radiotherapy. At this follow-up visit, red and white changes were note at the left buccal muosa (A) and left ventral tongue (B), mimicking erythroleukoplakia.

I.3.2. Histology, the gold standard for risk prediction

Clinical risk factors help clinicians to identify OPLs and estimate their cancer risk and decide when and where to biopsy. The biopsy sample is then sent to a pathology lab for pathological evaluation of cancer risk at the histological level. Currently, histological assessment is the gold standard for determining the cancer risk for premalignant lesions. This assessment is based on identification of the presence and degree of histological changes called "dysplasia." The World Health Organization has established the following criteria for histological diagnosis of oral dysplasia (1978):

1. Loss of polarity of the basal cells;
2. The presence of more than one layer having a basaloid appearance;
3. Increased nuclear/cytoplasm ratio;
4. Drop-shaped rete-ridges;
5. Irregular epithelial stratification;
6. Increased numbers and abnormality of mitotic figures;
7. The presence of 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. Loss of intercellular adherence; and
12. Keratinization of single cells or cell groups in the prickle cell layer.

Dysplasia is a histological term that describes varying degrees of abnormal epithelial changes, such as increased nuclear / cytoplasmic ratio, an increased rate of mitotic figures, cellular pleomorphism, and nuclear hyperchromatism. Architecturally and pathologically, dysplastic lesions are further divided into mild, moderate, and severe forms depending upon how much of the tissue is dysplastic. Mild dysplasia is a lesion in which the dysplastic cells are confined to the lower one third of the epithelium. Moderate dysplasia is a lesion in which the dysplastic cells are evident in about half the thickness of the epithelium. Severe dysplasia is a lesion in which the dysplastic cells have filled the lower two-thirds of the epithelial thickness. In carcinoma *in situ* (*CIS*), the dysplastic cells occupy the entire thickness of the epithelium (bottom to top changes) although the basement membrane is still intact (Lumerman *et al.,* 1995). Invasion of 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 and the degree of dysplasia are believed to have a huge impact on the malignant risk of the premalignant lesions. All studies to date have shown that leukoplakia with dysplasia are more likely to progress to oral SCC than those without dysplasia (Waldron and Shafer, 1975; Lumerman *et al.,* 1995). A large clinical study by Silverman *et al* (1984) found that during a mean follow-up period of 7.2 years, more than 36% of leukoplakia lesions with epithelial dysplastic features eventually underwent malignant transformation, whereas those leukoplakia without dysplasia only demonstrated a malignancy rate of 15%. The relationship of malignant risk and degree

of dysplasia is further demonstrated by studies from the uterine cervix and other systems and organs including the skin and respiratory systems (Boone *et al.,* 1992; Braithwaite and Rabbitts, 1999; Geboes, 2000; Pinto and Crum, 2000; Shekhar *et al.,* 1998). The cancer risk in moderate or severe dysplasias appears to be much higher that in mild dysplasia or hyperplasia. Taken together, all these findings make the histological evaluation of presence and degree of dysplasia the gold standard for predicting the malignant potential of premalignant lesions in a number of organs and systems, including the oral cavity.

Based on these criteria, a histological progression model has been established for oral cancer (Figure 2). In this model, oral cancers progress through hyperplasia and increasing degree of dysplasia, mild, moderate, and severe, to *CIS*, and finally break through the basement membrane to become SCCs. Severe dysplasia and *CIS* are usually grouped together as high-grade dysplasia, because both are late stage, preinvasive lesions with a high malignant risk. Furthermore, distinguishing between these stages is often difficult and does not appear to be of practical value in the management of oral mucosa (Pindborg *et al.,* WHO 1997).

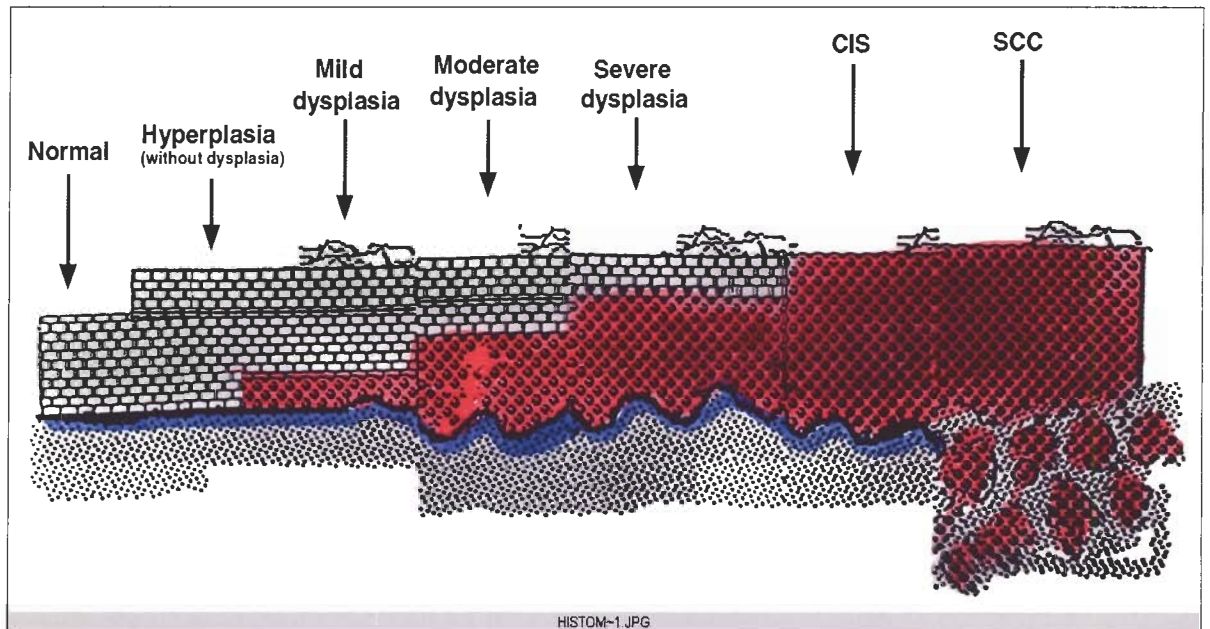


Figure 2. Histological progression model of oral premalignant and malignant lesions
A schema showing the well-established histological progression model of oral premalignant and malignant lesions. Oral SCC is believed to progress through a series of histological stages from normal tissue to hyperplasia and increasing degree of dysplasia, mild, moderate and severe, to carcinoma *in situ* (*CIS*), finally breaking through the basement membrane to become an invasive SCC.

I.3.2.1. Problems with the histological progression model

The histological progression model has a better predictive value for high-grade preinvasive lesions (severe dysplasia and *CIS*) than for low-grade lesions. In fact, many clinicians believe that high-grade lesions will inevitably become cancer if left untreated (Regezi *et al.,* 1989). As a result, high-grade preinvasive lesions are generally treated aggressively, and the histological progression model has served as a good guide for the aggressive treatment.

In contrast, the histological progression model is a poor predictor of malignant risk for lesions . with minimal or no dysplasia (i.e. those with hyperplasia without dysplasia and those with either mild and moderate dysplasias). This constitutes a major dilemma for clinicians. The majority of low-grade lesions do not progress (either remaining static or regressing), and only a small percentage do eventually develop into cancer. However, low-grade lesions constitute the bulk (~ 90%) of leukoplakia (hyperplasia without dysplasia, 80.1% and low-grade dysplasia, 12.2% compared with high-grade dysplasia, 4.5% and SCC, 3.1%) (Waldron and Shafer, 1975). Aggressive treatment does not seem to be justified for the majority of these lesions, both in terms of side effects and cost. Therefore, new methods to identify the small percentage of progressing low-grade lesions from the majority of non-progressing lesions are highly desired.

The significance of establishing these new methods lies in two aspects. First, they will facilitate the understanding of the mechanisms of early carcinogenesis. Second, they

will directly impact on the clinical management of these lesions. If we could understand the critical events occurring during early carcinogenesis, we might not only be able to predict the malignant potential at a very early stage but also be able to plan management of the small percentage of progressing lesions (e.g., aggressive treatment or chemoprevention). Hence, successful treatment of these early lesions and prevention of their progression is key to the management of this disease.

I.4. Molecular markers and loss of heterozygosity (LOH)

With the rapid expansion of molecular technology, it is now possible to study the mechanisms of oral carcinogenesis at a genetic level. This offers new approaches to identifying high-risk oral premalignant lesions and provides an opportunity for efficient intervention in patients harboring high-risk lesions at a stage in the disease where curative aims may be achieved for the majority of patients. This thesis employed recent front-edge molecular techniques to address some of the aforementioned problems, in the hope of defining at a molecular level the nature of the DNA alterations and developing useful approaches to reducing both morbidity and mortality of oral cancer.

I.4.1. Molecular mechanism of carcinogenesis

It is generally accepted that most sporadic tumors arise as a result of a multi-step process of accumulated genetic alterations. In 1976, Nowell proposed that neoplastic transformation occurred in a single stem cell that had a critical genetic alteration that gave it a growth advantage over its neighbor cells. This mutation resulted in an outgrowth of a clone of altered (initated cells), in which further mutation occurred. Over time, with the accumulation of more mutation and repeated clonal outgrowths alterations became apparent in epithelial cell behavior and structure, generating the clinical and microscopic alterations to histology that define premalignant stages and invasive lesions.

Early in 1990, a molecular model outlining discrete genetic events that occur in the progression of colon cancer from pre-cancerous polyps to invasive tumors was established by Fearon and Vogelstein. Many of these genetic events take place well before a given tumor produces clinical symptoms and often before a benign lesion or focus of dysplasia develops into an invasive cancer (Sidransky, 1997). Therefore, the identification of individual molecules that are associated with malignant transformation will lead to an increasing number of molecular markers that may be indicative for the risk of premalignant lesions and could be used as a more precise method for the early diagnosis of cancer. Although the precise order and number of events required for tumorigenesis remains unknown, a recent series of studies demonstrated six important steps that are believed to be necessary for a cancer to develop (Hahn *et al.,* 1999; Weinberg *et al.,* 2000). These steps include the following: 1) Acquisition of autonomous proliferative signaling; 2) Inhibition of growth inhibitory signals; 3) Evasion

of programmed cell death; 4) Immortalization; 5) Acquisition of a nutrient blood supply (angiogenesis); and 6) Acquisition of the ability to invade tissue.

The process of clonal evolution that gives rise to oral tumors is presented as a schematic in Figure 3.

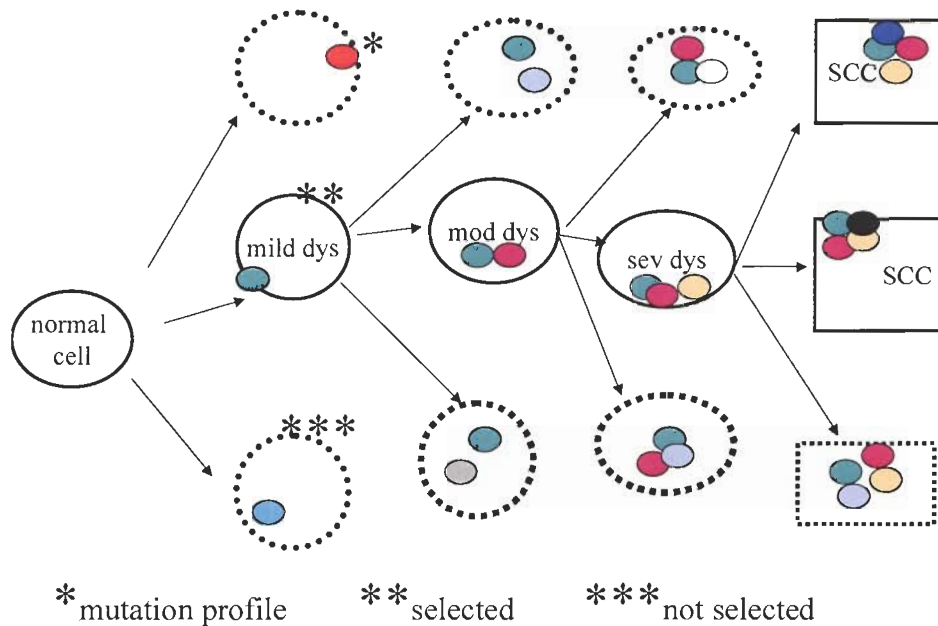


Figure 3. Clonal evolution during oral carcinogenesis.

A schematic depiction of the process by which clonal divergence occurs in an oral lesion. The gradual accumulation of critical mutations essential for tumour development in a clone of cells is shown with the symbol *. Cells labeled ** are those that have the greatest survival advantage over surrounding cells, allowing them to become predominant clones that expand and take over the epithelium. In contrast, cells designated with *** are those that undergo apoptosis, and so are lost to the tissue.

I.4.2. Oncogenes and tumor suppressor genes (TSGs)

The notion of cancer being a consequence of the accumulation of a series of genetic alterations has led to the identification of a number of critical gene alterations in carcinogenesis. In the head and neck region, 7-10 independent genetic events are believed to be involved in the production of invasive SCC (Renan, 1993). These genetic alterations involve three major types of genes: genes that preserve the integrity of the genome, oncogenes, and tumor suppressor genes (TSGs). Oncogenes and TSGs, and the proteins for which they code, act antagonistically to control the processes of cellular proliferation and differentiation in a normal tissue. Therefore, they are thought to be the most important gene classes that drive carcinogenesis at a molecular level (Bishop 1991 and Vogelstein, 1992).

Oncogenes were originally identified as the transforming genes in retroviruses. They are activated forms of normal, non-activated cellular genes, termed proto-oncogenes, which are produced by genetic change in either their coding region or regulator sequences. The products of proto-oncogenes play a key role in regulating the cascade of events that control normal growth, differentiation, and mortality of cells.

In human cancers, proto-oncogenes are frequently located adjacent to chromosomal breakpoints and often identified in metaphase spreads of cancers at chromosomal translocation events. In addition to translocation, various other mechanisms have been

described for activation of these proto-oncogenes including point mutation, truncation, and gene amplification (Vogelstein and Kinzler, 1998). Only one of the two gene copies needs to be changed for an effect to be observed for an oncogene. This is referred to as a "dominant gain-of-function" mutation. Presently nearly 50 known different proto-oncogenes have been identified, coding for proteins that function as growth factors, growth factor receptors, cytoplasmic second messengers, protein kinases, nuclear phosphoproteins, transcription factors, and others. They can be roughly subdivided into two groups. One class of genes rescues cells from senescence and programmed cell death, acting as immortalizing genes. A second class of genes reduces growth factor requirements and induces changes in cell shape that results in a continuous proliferative response (Vogelstein and Kinzler, 1998). The activation of these proto-oncogenes to oncogenes can contribute to the release of cells from the normal controls of proliferation, death, migration, and adhesion to cause neoplastic transformation.

In contrast to oncogenes, tumor suppressor genes (TSGs) are a group of genes encoding proteins, which, through a variety of mechanisms, function to negatively regulate cell growth and differentiation pathways. According to Knudson's hypothesis (1985), both copies of a tumor suppressor gene have to be inactivated for its protective function to be completely lost in a cell; hence, such alterations are said to be "recessive, loss-of-function" mutations. Experience with known suppressor genes, such as the retinoblastoma gene, suggests that this process involves two separate events, the first quite often involving a point mutation in one allele, followed by loss of the locus

containing the wild type gene in the remaining allele (loss of heterozygosity, LOH). It has been widely accepted that functional loss of TSGs is one of the most common genetic alterations during carcinogenesis (Leis *et al.,* 1996).

To date, approximately 50 tumor suppressor genes have been localized and identified in a number of cancer types, including *p53, RB* (retinoblastoma), *VHL* (the gene responsible for von Hippel-Lidau syndrome*), FHIT* (Fragile histidine triad)*, p16, DPC4, APC (*adenomatous polyposis coli)*, doc-1* (deleted in oral cancer)*, TSC2, BRCA1, NF-1, NF-2 and WT-1* (Mao *et al.,* 1996 and 1998; Reed *et al.,* 1996; Gleich *et al.,* 1996; Todd *et al.,* 1995; Largey *et al.,* 1994; Pavelic *et al.,* 1997; Uzawa *et al.,* 1994; Kim *et al.,* 1996; Latif *et al.,* 1993; Kanno *et al.,* 1994 and Sparks *et al.,* 1998). Although the cellular functions of tumor suppressor proteins, such as p105-RB, p53 and p16, are becoming increasingly well understood, others remain largely undefined. It is clear, however, that the tumor suppressor proteins exhibit a variety of functions within the cell. Some tumor suppressor proteins have been shown to directly or indirectly antagonize the function of proto-oncogenes in growth regulation.

A rather simplistic analogy can be made between the functions of these classes of genes in a cell and driving a car. Proto-oncogenes function as accelerators that cause the cell to divide and grow. By contrast, the tumor suppressor genes normally function to restrain the growth of the cell, much like the brakes of a car stop it from moving forward. 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 during a multistep process in which a cell accumulates many genetic changes, eventually leading to a dysregulation of cell growth and the induction of a malignant phenotype. Therefore, studies of oncogenes and TSGs not only define the multi-step carcinogenetic process but also lay the groundwork for developing prognostic indicators for tumor progression that could impact significantly on the clinical management of cancers and premalignant lesions.

I.4.3. Oncogene and TSG in oral premalignant and malignant lesions

Few oncogenes have been identified in head and neck squamous cell carcinoma (HNSCC), although changes in the expression of many genes have been reported. Among accepted oncogenes are *ras, cyclin-D1, myc, erbB, bcl-1, int-2, CK8* and *CK19* (Kiaris *et al.,* 1995; Lese *et al.,* 1995; Saranath *et al.,* 1993; Warnakulasuriya *et al.,* 1992; Wong *et al.,* 1993; Bartkova *et al.,* 1995; Xu *et al.,* 1995; Masuda *et al.,* 1996 and Riviere *et al.,* 1990). Our knowledge of the frequency of mutation of these genes in different populations is still somewhat limited. For example, *ras* and *myc* mutations appear to be more prevalent in head and neck tumors occurring in the Far East, possibly due to the use of chewing tobacco and betel quid by these populations (Anderson *et al.,* 1994; Clark *et al.,* 1993; Paterson *et al.,* 1996 and Saranath *et al.,* 1993). Mutations of H-*ras* can be identified in approximately 35% of tumors in the latter group; however, the prevalence of these mutations in Western patients is only five per cent (Kiaris *et al.,* 1995; Matsuda *et al.,* 1996 and Sakata, 1996). In addition, very few studies have included an analysis of mutation frequencies in premalignant lesions. The few studies available tend to use

immunohistochemical analysis and look at increased expression of the gene, not mutation. For example, Hou *et al.,* (1992) reported a progressive increase in *c-erb-2/neu* expression as premalignant lesions advanced to malignant lesions. However, it is not known whether this effect was due to a mutation of the gene itself or to a dysregulation of the expression of this gene resulting from a downstream effect of another mutation.

On the other hand, many studies have focused on the role of TSGs in oral carcinogenesis. Some of the TSGs involved in head and neck cancers include *p53, Rb* (*retinoblastoma*), and *p16INK4A* (Gallo *et al.,* 1999; Gleich *et al.,* 1996; Jares *et al.,* 1999; Liggett *et al.,* 1996; Papadimitrakopoulou *et al.,* 1997; Partridge *et al.,* 1998 and 1999; Pavelic and Gluckman, 1997; Reed *et al.,* 1996; Sartor *et al.,* 1999). Other potential candidates are *FHIT* (Fragile histidine triad), *APC* (adenomatous polyposis coli), *doc-1* (deleted in oral cancer), *VHL* (the gene responsible for von Hippel-Lidau syndrome) and *TβR-II* (the gene coding for transforming growth factor type II receptor). (Croce *et al.,* 1999; Largey *et al.,* 1994; Mao *et al.,* 1996; Mao, 1998; Todd *et al.,* 1995; Uzawa *et al.,* 1994 and Waber *et al.,* 1996).

Recent advancement in molecular analysis techniques has rapidly revolutionized our ability to look at these genetic alterations. Most studies on TSGs, particularly those in oral premalignant lesions, use microsatellite analysis to identify loss of heterozygosity (LOH) in DNA extracted from epithelial cells belonging to these lesions. This is also the major technique used in research described in this proposal.

I.4.4. Loss of heterozygosity (LOH) studies using microsatellite analysis

LOH, loss of heterozygosity, has been shown to be a powerful molecular technique for identifying genetic alterations to tumor suppressor genes. It can detect changes as small as a few thousand nucleotides in size, up to whole chromosome loss or gains. The LOH assay is designed to assess polymorphic chromosomal regions that map close to or within putative or known TSGs. The concept of LOH is consistent with Knudson's two-hit hypothesis, which states that inactivation of one of the two alleles of tumor suppressor genes by either a germline or somatic mutation is a critical step in carcinogenesis because only one more mutation inactivation is required to the remaining allele before expression of the phenotype occurs (Knudson, 1985). LOH analysis has been used in the discovery of some important genes, including *APC* (adenomatous polyposis coli) gene, *DCC* gene, *DPC4* gene and *ATM* gene, etc.

Two methods are available for the study of LOH: restriction fragment length polymorphism (RFLP) analysis and microsatellite analysis. The advantages of using microsatellite markers in LOH assay are many fold. 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 RFLP analysis, which is based on base substitutions at endonuclease recognition sites. Second, this [α-P^{32}] end-labelled PCR-based approach is much more sensitive than RFLP analysis, requiring only a small amount of DNA (5 nanograms or less per reaction), which is critical for the study of premalignant lesions. Another advantage is that

microsatellite makers can be used in paraffin-embedded archive samples, in addition to fresh or frozen samples, which is critical for a retrospective study of samples. For these reasons, this proposal employs microsatellite analysis.

Microsatellites contain runs of short and tandemly repeated sequences of di-, tri-, or tetra-nucleotides, such as –GTGTGT-, -GTAGTAGTA-, or –GTACGTACGTA-(Figure 4). These short repetitive DNA sequences are called short tandem repeats (STRs) or microsatellites. The number of such tandem repeats is found to be highly polymorphic in the population, with individuals often containing 2 different alleles for a given region, with a different number of copies of the repeats (generally 4 to 40) in them (NIH/CEPH, 1992). In addition, they are well interspersed throughout the human genome (e.g., estimated every 30-60 kilo base pairs (kb) for CA repeats) and are highly conserved through successive generations (Ah-See *et al.,* 1994; Beckman and Weber, 1992). 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 (Weber and May 1989). The basic rationale for use of this assay is that a frequent finding of a loss in a particular segment of a chromosome in a tumor type is highly suggestive of the presence of a critical tumor suppressor gene within this region. Loss of heterozygosity suggests that a putative tumor suppressor gene nearby also may be lost. The detection of loss of one allele in the clinical sample demonstrates the presence of a clonal population of cells that share altered genetic information, a characteristic of cancer cells (Cairns and Sidransky, 1999).

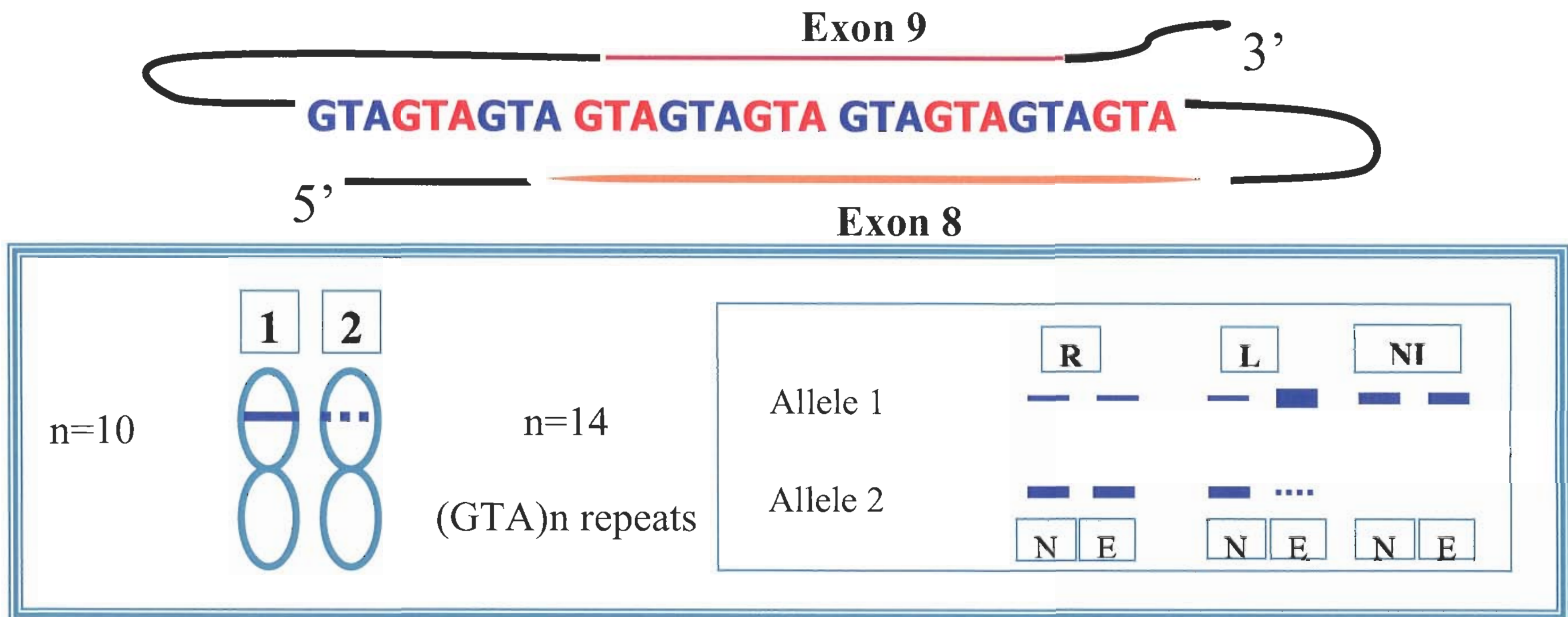


Figure 4. Microsatellite analysis to identify LOH.

Figure 4 illustrates the general mechanism of microsatellite analysis which is used to identify LOH in this thesis. The prerequisite for using this techinique is that the number of repeats of certain region of a chromosome is different between two parental alleles. In the illustration above, the alleles contain 10 and 14 repeats of the nucleotide sequence GTA. PCR amplification of this region creates to different sized fragments that can be separated on acrylamide gels. The figure also illustrates the 3 types of patterns observed with this analysis: retention of both alleles (R), loss of one allele (L) and noninformativity (NI, patient is homozygous for the allele and hence cannot be evaluated with this marker). These patterns are determined by comparing the relative signal intensity between the separated two bands (proportion) in a patient's normal and tumor tissue.

I.4.5. The significance of studying LOH in cancer research

Information obtained from LOH studies has two merits. The finding of frequently lost regions during cancer development can lead to discovery of new TSGs. Such was the case for several important TSGs, like *Rb* and *MEN1* (Iwasaki, 1996; Yokoyama, 1996). LOH analysis can also be used to obtain critical information on the role of the presumptive TSGs in cancer development, even prior to the identification of the actual TSG. For example, 3p loss is one of the most common events in both OPLs and oral SCC, as well as in premalignant and malignant lesions of a number of other organs (Deng *et al.,* 1998; Euhus *et al.,* 1999; Guo *et al.,* 1998 and Maestro *et al.,* 1993). Three discrete regions of deletion have been found for oral SCC and each is suspected to contain at least one TSG. One tentative TSG, *FHIT* (Fragile histidine triad), has been located at 3p14, although acceptance of this gene as a TSG is still controversial. TSGs at the other deletion regions on 3p may also play important roles in cancer development, although these genes have yet to be identified.

I.4.6. LOH studies of oral cancers

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 (Ah-See *et al.,* 1994; Nawroz *et al.,* 1994 and Rosin *et al.,* 2000). There is, however, a relative lack of studies of genetic changes in oral premalignant lesions which further

progressed into oral SCC. This partly results from the fact that accession to oral premalignant lesions is less easy as compared to access to oral SCC in big research hospitals, and partly from the fact that oral premalignant lesions are generally much smaller than SCCs, which makes it relatively harder to get sufficient DNA for analysis.

In this thesis, microsatellite markers on chromosome arms 3p, 4q, 8p, 9p, 11q, 13q, and 17p will be used to evaluate the risk of SOM development of premalignant lesions, since they have been previously reported to be associated with progression of oral premalignant lesions and possibly could be used as risk indicators in each step of the progression of oral cancer (Rosin *et al.,* 2000). Each of these regions will be discussed as follows.

Chromosome 3. High frequency of LOH at chromosome 3p has been reported in head and neck cancers. The losses appear to center around 3p13-21.1, 3p21.3-23, and 3p24-25 (Maestro *et al.,* 1993 and Wu *et al.,* 1994). The 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 sites, called FRA3B, in the human genome. Fragile sites are portions of chromosomes that are extremely weak and break easily. Consequently, carcinogens such as those found in tobacco may easily target these weak areas. The gene, *FHIT* (Fragile histidine triad) appears to be involved

in various cancers including esophageal, gastric, colonic, mammary, cervical, small cell lung, and head and neck carcinomas (Mao *et al.,* 1996; Pennisi 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 cells in the absence of the normal expression of the gene and may eventually lead to dysregulated DNA synthesis and cell replication (Mao *et al.,* 1996).

Until now there is sufficient evidence for only one gene, *FHIT*, as a candidate TSG in the region 3p14.3, although the evidence in support of it being a TSG is still considered controversial (Mao, 1998; Gonzalez *et al.,* 1998). TSGs that are responsible for LOH at the other two regions (3p24-pter, and 3p21.3) are still not identified. For example, the 3p24-25 region 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). Its alteration has been reported in cancers, especially in those that are *VHL*-associated (Decker *et al.,* 1997; Kok *et al.,* 1997; Van den Berg and Buys, 1997). Uzawa *et al.,* (1998) mentioned the possibility that the *VHL* gene may in involved in oral SCC development. However, mutations of the *VHL* gene could not be identified and the examination of this gene for other methods of inactivation, such as by hypermethylation, has yielded negative results. It is possible that allelic loss of chromosome arm 3p in HNSCC involves regions surrounding the *VHL* locus but not the

VHL gene itself. Another TSG in HNSCC may exist in the regions surrounding D3S1110 at 3p25 (Uzawa *et al.,* 1998; Waber *et al.,* 996).

Chromosome 4. LOH on chromosome 4 has been studied in cancers of many organs including liver, bladder, ovary, and uterine cervix. The putative tumor suppressor locus has been localized to a region near the epidermal growth factor (*EGF*) locus at 4q25. Loss at 4q25 occurs in 75% of head and neck cancers (Pershouse *et al.,* 1997) and loss at 4q24-26 occurs in 47% (Bockmuhl *et al.,* 1996 and Califano *et al.,* 1996). The combination of allelic deletions and chromosomal transfer studies strongly suggests the presence of a TSG within 4q24-26. In total, LOH at this region was involved in >80% of the tumors examined, strongly suggesting that a putative TSG(s) on chromosome 4q 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-See *et al.,* 1994; Bockmuhl *et al.,* 1996; Califano *et al.,* 1996; el-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 (el-Naggar *et al.,* 1995 and Wu *et al.,* 1997). Several studies have linked allelic loss at 8p to a higher clinical stage (Wu *et al.,* 1997) and poorer prognosis (Li *et al.,* 1994 and Scholnick *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 the majority of malignant lesions. The most commonly affected region is chromosome 9p21-22. At 9p21, the prime TSG candidate involved in the head and neck cancers is cell cycle gene *p16* (also know as *MTS-1* for major tumor suppressor-1, *INK4a* for inhibitor of cyclin-dependent kinase 4a, and *CDKN2A* for cyclin-dependent kinase inhibitor 2A). *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 biochemical effect of p16 is to halt cell-cycle progression at the G1/S boundary. Approximately 80% of the head and neck cancers and premalignant lesions were p16 inactivated at the protein and/or DNA level, suggesting that inactivation of p16 may play an important role in early head and neck cancer development (Papadimitrakopoulou *et al.,* 1997 and Reed *et al.,* 1996). Mutations of this gene are not apparently frequent for oral cancer (Dawson *et al.,*, 1996; Reed *et al.,*, 1996). The gene is inactivated by alternative mechanisms including homozygous deletion and 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). Alternatively, another tumor suppressor gene may exist in this region (Dawson *et al.,* 1996; Reed *et al.,* 1996 and Waber *et al.,* 1997) which may play a role in aggressive disease as manifest by local, regional, or distant recurrence (Lydiatt *et al.,* 1998 and Matsuura *et al.,* 1998).

Chromosome 11. LOH on human chromosome 11 has also been commonly reported in a variety of cancers, including HNSCC (39%-61%) (Bockmuhl *et al.*, 1996; Califano *et al.*, 1996; el-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 at 11q13 (Nawroz *et al.*, 1994). 11q13 is a region that harbors several proto-oncogenes, such as *INT2*, *bcl-1*, *Cylin D1*, and *FGF*. It is possible that some of this region's allelic imbalance may be due to amplification rather than LOH (Nawroz *et al.*, 1994), since the "loss" at 11q13 approximates the known percentage of 11q amplification in HNSCC (Nawroz *et al.*, 1994 and Somers, 1990). Amplification of this region in association with poor prognosis has also been reported (Meredith *et al.*, 1995 and Papadimitrakopoulou *et al.*, 1997). In addition, loss at 11q23, another hot spot in the long arm of chromosome 11, was found in association with a higher likelihood of recurrence of HNSCC (Lazar *et al.*, 1998).

Chromosome 13. More than half of HNSCCs show LOH of 13q in regions near to the *RB* (retinoblastoma) locus, but not at *RB* gene (52-67%) (Bockmuhl *et al.*, 1996; Califano *et al.*, 1996; Nawroz *et al.*, 1994 and Ogawara *et al.*, 1998). A hot spot at *D13S133* at 13q14.3, which lies just telomeric to the RB gene, has been reported (Yoo *et al.*, 1994). Recent studies showed LOH on 13q14.3 to be significantly correlated with lymph node metastasis for oral cancer and esophageal SCC (Harada *et al.*, 1999 and Ogawara *et al.*, 1998). Some data suggest another unidentified TSG(s) in region 13q21 might also be involved (Soder *et al.*, 1995).

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 and 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 (Lazarus *et al.,* 1995). The p53 protein functions as a mediator in several activities, including transcription activation, DNA repair, apoptosis, senescence, and G1 and G2 cell cycle inhibition. In addition, there is increasing evidence for another novel TSG at a region, defined by the cholinergenic receptor B1 (*CHRNB1*) locus at 17p11.1-12, that is tightly linked to the *p53* regions (Adamson *et al.,* 1994 and el-Naggar *et al.,* 1995).

Fractional allele loss (FAL). Vogelstein first defined FAL in a tumor as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative (Vogelstein *et al.,* 1989). FAL can provide information concerning the genetic burden of the disease during its progression as measured by clinicopathological parameters and survival data. In addition, the results of such detailed allelotypes may aid the interpretation of carcinogenesis and development of molecular progression models for specific tumors. The first comprehensive allelotype study of HNSCC, which analyzed 52 oral cancers, showed that a "FAL > median (0.22)" group is correlated with nodal involvement and poor survival (Field *et al.,* 1995). A more recent study of FAL also showed that allelic imbalance at 3p22-26, 3p14.3-12.1 and 9p21 was a better prognosticator than the TNM system (Partridge *et al.,* 1999).

I.4.7. LOH analysis 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 oral SCC. However, to date, few studies have investigated the genetic profiles at premalignant stages of the oral lesions. The main difficulties lie in the fact that: 1) premalignant lesions are small and, therefore, it is extremely hard to obtain sufficient DNA for molecular analysis, 2) big hospitals or research centers typically have better access to cancers than to premalignant lesions. In addition, the limited number of studies either used only a small number of cases or primers, or did not correlate LOH with degree of dysplasia. Nonetheless, results from these studies clearly show that LOH is a frequent event in premalignant lesions (Califano *et al.,* 1996; el-Naggar *et al.,* 1995; Emilion *et al.,* 1996; Mao *et al.,* 1996 and Roz *et al.,* 1996). For example, a similar frequency of LOH at 9p was reported in preinvasive lesions (71%) as in SCCs (72%) (van der Riet *et al.,* 1994). This suggests that loss of 9p is an early event in the progression of oral cancer (Papadimitrakopoulou *et al.,* 1997 and van der Riet *et al.,* 1994). Similarly, LOH at 3p has been found to occur very early during oral carcinogenesis and in a significant number of oral mild dysplasia or even hyperplasia (Zhang *et al.,* 1997). A few studies have shown that LOH at 3p14 and 9p21 in oral premalignant lesions may have prognostic significance for malignant progression of premalignant oral lesions (Hu *et al.,* 1996; Mao *et al.,* 1996; Patridge *et al., 1996*; Rosin *et al.,* 2000 and Zhang *et al.,* 1997). On the other hand, data from this lab showed that

LOH at 17p was not found in reactive hyperplastic lesions and mild dysplasia of oral mucosa, indicating loss at 17p occurs later than LOH at 3p and 9p (Zhang *et al.,* 1997).

Meanwhile, El-Naggar and his colleagues (1998) recently found LOH at 8p in 27% of dysplastic lesions and in 67% of invasive oral and laryngeal SCCs. The highest frequency of allele losses in dysplasia and cancer were detected in the same loci: 8p21 and 8p22. In addition, allelic losses in both dysplastic and corresponding invasive specimens were noted at the same loci, suggesting their emergence from a common preneoplastic clone (Califano *et al.,* 2000). These studies suggested that inactivation of TSG(s) within these loci may constitute an early event in the evolution of oral SCC.

I.4.8. A molecular progression model for oral cancer

Fearon and Vogelstein were the first ones to propose a molecular progression model for colorectal cancer, in 1990 (Fearon *et al.,* 1990). Later, molecular progression models were proposed in other tissues and organs. In a hallmark study by Califano *et al.,* (1996), allelic loss was investigated in the whole spectrum of premalignant and malignant lesions of head and neck region, including hyperplasia, dysplasia, CIS, and SCC. The study proposed a molecular progression model for HNSCC (Califano *et al.,* 1996). The model proposed that 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, 8, 11q, 13q, and 14q.

However, the study merged all dysplasias (mild, moderate and severe) into one study group. It is well accepted that with increasing degrees of dysplasia there is an increasing risk of malignant transformation. While the majority of mild dysplasia will not progress into cancer, severe dysplasia, similar to *CIS*, has a much higher probability of cancer progression. Therefore, figuring out the different genetic profiles at different stage of dysplasia will enable us to better understand the progression pathway of oral cancer at molecular level.

A recent study in this lab (Rosin *et al.,* 2000) has further refined this molecular progression model for oral SCC by investigating all degrees of oral dysplasias by means of LOH analysis using multiple microsatellite primers for the seven chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q, and 17p) known to be frequently lost in oral tumors (Figure 5).

The data supported the findings from previous studies that accumulations of genetic changes are critical for tumor progression, and further proposed that specific patterns of allelic loss occurred at different degrees of dysplasia.

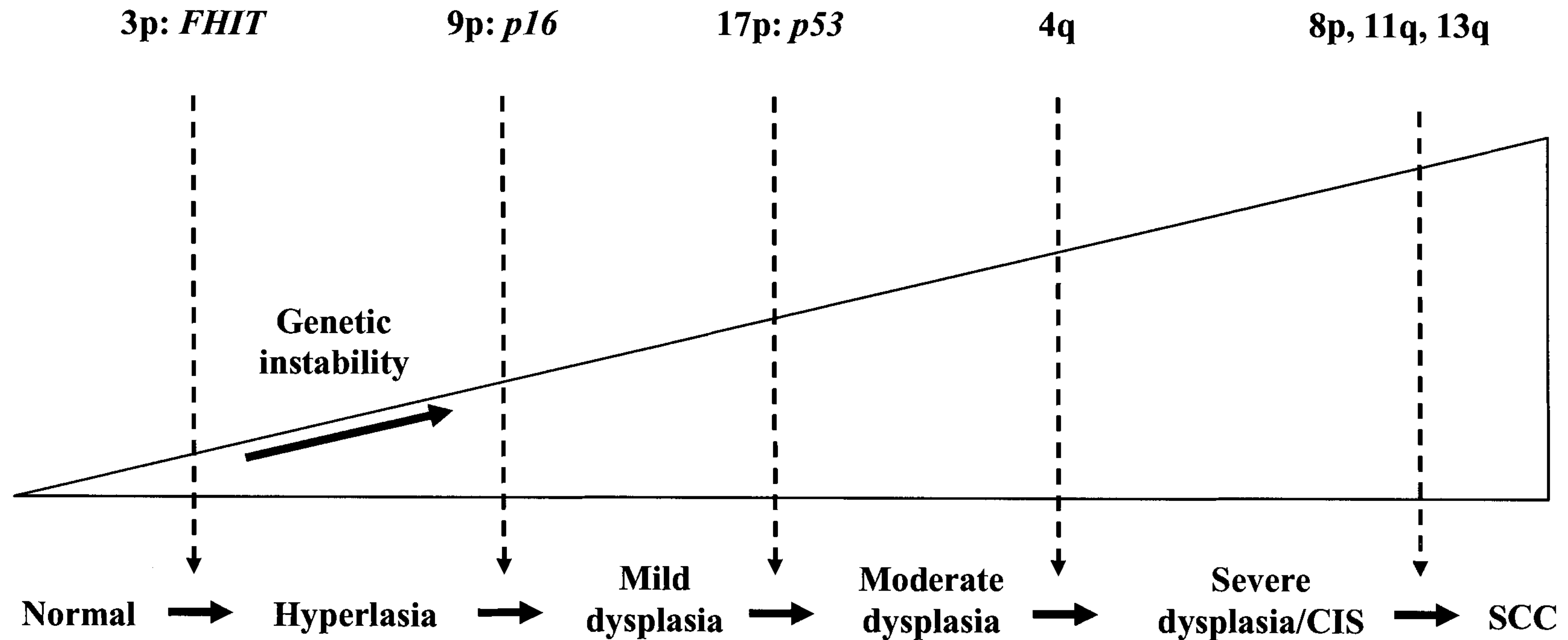


Figure 5. Molecular progression model for oral SCC.
Schematic demonstrating the genetic progression model of oral cancer established in our laboratory using LOH analysis. Oral cancer develops through a series of genetic events that parallel the histopathological progression of a neoplasm as it develop through a sequence of premalignant stages and finally into an invasive tumor. Different genetic changes are associated with different stages of oral cancer development: 1). 3p loss is associated with lesions with low-risk morphological alterations, i.e. hyperplasia and mild dysplasia; 2). The most common changes seen in moderate-risk histological stages (mild and moderate dysplasia) are 3p, 9p, and 17p; and 3). 4q loss is often associated with the transition between moderate and severe dysplasias/*CIS*; 4). Losses at 8p, 11q, and 13q are significantly increased in SCC compared to severe dysplasias/*CIS*.

I.5. LOH as risk predictors for OPLs

I.5.1. LOH as risk predictors for primary OPLs

The first study of LOH as a risk predictor is by Mao *et al.,* in 1996, and they found that OPLs with LOH at 9p21 and/or 3p14 cancer increased risk of malignant transformation: Thirty-seven percent of OPLs with LOH progressed into cancer, compared to only 6% of lesions without LOH. Similarly, a study by Partridge *et al.,* (2000) showed that 94% of hyperplasia and dysplasia lesions with 9p and/or 3p losses progressed into SCC.

In an article from our laboratory, Rosin *et al.,* (2000) examined LOH at 3p, 4q, 8p, 9p, 11q, 13q, and 17p in hyperplasia, mild, and moderate dysplasias in patients with or without progression into cancer. The progressing group acquired more LOH than its counterpart. Ninety-seven percent of the cases in this group exhibited loss at 9p and/or 3p, with additional losses noted at higher frequency on other arms. Using the data generated in this study, a model was proposed which placed individuals with premalignant lesions into 3 categories of risk depending on LOH patterns: 1) low risk: retention at 3p and 9p; 2) intermediate risk: loss at 3p and/or 9p; and 3) high risk: loss at 3p and/or 9p plus 4q, 8p, 11q, 13q, or 17 p. The highest risk group had a 33-fold increase in cancer risk compared to the low risk group. The study also suggests that for a majority of cases, alterations at 3p and/or 9p LOH may be a prerequisite for malignant transformation, since it is highly associated with the malignant transformation. These

studies suggest that LOH could be an excellent marker for cancer prediction since it can differentiate the clinically and histologically similar progressing lesions from those non-progressing ones with low risk.

I.5.2. LOH as risk predictors for OPLs in patients with a history of oral cancer

A recent retrospective study from this lab has investigated the utility of LOH as a marker to identify OPLs at high risk for progression into SOM (Rosin *et al.*, 2002). The LOH data on chromosomes 3p, 4q, 8p, 9p, 11q, 13q and 17p in leukoplakia lesions at former cancer sites (with known outcome, some of them progressing to SOM, some not) showed that 3p and /or 9p loss in these post-treatment leukoplakias was associated with a 26.3-fold increase in the risk of developing SOM compared with those that retained both of these arms. The significant LOH information collected in this study represents a possible use of 3p and 9p loss in post-treatment lesions as a simple and direct test for stratifying risk of SOM development. The data strongly suggest that the identification of such alterations at a former cancer site should alert the clinician to the presence of a potentially aggressive lesion, even if the histological diagnosis is hyperplasia or mild dysplasia, and even if distinction between SPT and recurrence could not be determined.

Table I-3. LOH and SOM

	SOM (%) (n = 36)	Non-SOM (%) (n = 32)	*P*
No. with LOH [a]	35 (97)	15 (47)	***0.0001***
1 arm lost	26 (72)	9 (28)	***0.0006***
LOH on 3p	22/35 (63)[b]	3/32 (9)[b]	***0.0001***
LOH on 9p	28/36 (78)	9/32 (28)	***0.0001***
LOH on 4q	12/30 (39)	1/26 (4)	***0.001***
LOH on 8p	13/36 (36)	6/32 (19)	*0.180*
LOH on 11q	6/32 (19)	1/31 (3)	*0.100*
LOH on 13q	4/32 (13)	2/30 (7)	*0.670*
LOH on 17p	13/36 (36)	9/32 (28)	*0.610*
LOH on 3p and/or 9p (but no other arms)	11/36 (31)	3/32 (9)	***0.039***
LOH on 3p and/or 9p (plus LOH at any other arm)	24/36 (67)	7/32 (22)	***0.0003***
All cases with LOH on 3p and/or 9p	35/36 (97)	10/32 (31)	***0.0001***

[a] A total of seven chromosomal arms were tested. Values in parentheses are percentages.
[b] Loss/informative cases (% loss)
Adapted from Rosin et al., (2003).

I.6. Exfoliative cells obtained non-invasively for LOH analysis and cancer prediction

One of the main barriers to the use of molecular techniques to identify and manage OPLs is the requirement for biopsies to provide the specimens for analysis. This is particularly a problem when clinicians are reluctant to repeatedly biopsy fragile sites of previous oral cancer or when there is no obvious clinical lesion. In patients with a

history of oral cancer, it is well known that the site of previous cancer is a high risk site with or without clinical apparent lesions. A possible solution to this problem is to perform the molecular analysis on exfoliated cells collected non-invasively from a tissue.

I.6.1. Exfoliative cytology

Oral biopsy represents the gold standard for determining the nature of a mucosal lesion and for diagnosing SCC, and exfoliative cytology has, until recently, been discounted as a tool for assessing oral mucosal lesions. However, techniques have now been reported that include evaluation of exfoliated oral epithelial cells and comparisons of these methods with biopsy techniques. Exfoliative techniques have the advantage of being minimally invasive, and they do not require local anesthetic. Technically, full-thickness sampling is essential if histomorphologic evaluation of the collected cells is to yield representative findings. For example, many dysplastic lesions are first identified in the basal epithelial layers, and the diagnostic histomorphologic findings may be lost as the cells mature and parakeratin and keratin are produced. In the past, exfoliated cell collections did not sample the full thickness of the epithelium, which led to a large number of false-positive and false-negative results, relative to diagnosis by biopsy. However, recently, use of a cytobrush reportedly allows sampling of the full thickness of stratified squamous epithelium of the oral mucosa (Sciubba *et al.,* 1999) and therefore yields more complete information about the tested region. Molecular studies about various types of cancers have proved that combining information from molecular markers with exfoliative techniques are sensitive and specific procedures that can be performed

sequentially over time and perhaps as screening methods for at-risk lesions already identified (Epstein *et al.,* 2002).

I.6.2. Evidence supporting the use of exfoliated cells for tumor risk assessment

A few recent studies examining the feasibility of analyzing and monitoring clonal genetic changes using this approach have demonstrated the capability of cytological brushings of different types of neoplasms to produce cells with tumor-specific LOH defined by microsatellite alterations, and these changes observed in exfoliated brushing cells closely represent those in biopsy specimens of same region (Brennan *et al.,* 1991; Mao *et al.,* 1994 and 1996; Spafford *et al.,* 2001). Furthermore, the examination of exfoliated cells for molecular markers may also allow assessment of the progression of change and the outcome of therapy, including preventive studies (Mao *et al.,* 1998). Our laboratory has investigated whether exfoliated cells taken from a specific site in the oral cavity could reveal molecular changes present at that site. LOH of biopsies of oral SCCs were compared to LOH from exfoliated cell samples collected by scraping the same SCC prior to biopsy. The results showed that LOH patterns in exfoliated cells are highly representative of those in biopsies (Rosin *et al.,* 1997) (Table I-4). In summary, taken together, all these studies have shown that allelic loss can be identified in exfoliative cells and that the profile of change in these cells is similar to that observed in biopsies, which makes it plausible to take multiple samples easily and non-invasively during follow-up for oral cancer research.

This study was designed to validate the use of exfoliative cells for LOH analysis and cancer prediction within an ongoing prospective study of oral cancer patients in long-term follow up.

Table I-4. Microsatellite analysis of exfoliated cells and concurrent biopsies

Patient	Age(yr)/Sex	Diagnosis	Chromosomal arms showing LOH	
			Exfoliated cells	Biopsy
1	44/F	Mild dysplasia	No loss	No loss
2	75/M	Mild dysplasia	No loss	No loss
3	58/F	Moderate dysplasia	9p	9p
4	80/F	Moderate dysplasia	9p	9p
5	37/F	Severe dysplasia	3p, 9p, 17p	3p, 9p, 17p
6	60/M	Severe dysplasia	No loss	No loss
7	79/F	CIS	9p	9p
8	64/F	SCC	3p	3p, 9p
9	64/M	SCC	9p, 17p	9p, 17p
10	74/F	SCC	3p	3p
11	46/M	SCC	3p, 9p, 17p	3p, 9p, 17p
12	55/F	SCC	3p, 9p, 17p	3p, 9p, 17p
13	54/F	SCC	9p, 17p	9p, 17p
14	41/M	SCC	9p	9p
15	89/F	SCC	3p, 9p, 17p	3p, 9p, 17p
16	47/F	SCC	9p, 17p	3p, 9p, 17p
17	70/M	SCC	3p	3p, 9p, 17p
18	53/M	SCC	3p, 17p	3p, 17p
19	63/F	SCC	3p, 9p, 17p	3p, 9p, 17p
20	30/F	SCC	3p, 17p	3p, 9p, 17p

I.7. Second oral malignancy (SOM) and its molecular basis

In spite of the significant advances in surgery and radiotherapy in upper aerodigestive tract cancer therapy as well as the intensive follow-up over the last decades, the mortality rate for oral SCC has been remained unchanged (5 year survival rate < 50%), which is mainly caused by the frequent development of a second oral malignancy (SOM) presented as either local regional recurrence or second primary tumor (SPT) (Lippman and Hong 1989; Mashberg *et al.,* 1989; Silverman *et al.,* 1990; Vikram *et al.,* 1994; Dhooge *et al.,* 1998). This poor outcome is largely due to a lack of sensitive diagnostic tools for monitoring patients after treatment. The current golden standard used to predict the risk of SOM still relies heavily on clinical and histopathological parameters, which are often difficult to assess for therapeutic decisions. Early detection of SOM is critically important for improving the survival rate and there is a persistent clinical need to improve restaging procedures to exclude or verify SOM at stages that allow for a successful therapeutic intervention.

Recent molecular studies have begun to describe the molecular basis behind "field cancerization" in the head and neck region, with many genetic alterations being identified adjacent to premalignant and malignant lesions. This theory will be discussed below. These findings enable us to better understand the molecular mechanism by which SOM occurs and progresses. The basis for these studies is the use of molecular procedures to trace clonal relationships among the different oral lesions or tumors in an individual,

allowing us to better differentiate local recurrence and SPT. Such information is critical to the development of alterative means of early prevention or therapy for patients with a history of oral cancer.

I.7.1. Recurrence of oral cancer

Recurrence of oral cancer is always a life-threatening event, leading to death in the majority of patients. After treatment of an oral cancer, tumor recurrence rates vary from 18 to 76% in the literature (Shah *et al.,* 1976; Jones *et al.,* 1992; Mishra *et al.,* 1996). The site of previous primary carcinoma has been widely recognized to be at a high risk for the development of recurrence with or without clinical apparent lesions.

A definition most often accepted for local recurrence by most investigators relies on clinical criteria time and distance, specifically the development of another carcinoma within 3 years and < 2 cm away from the primary carcinoma (Braakhuis *et al.,* 2002). Current golden standards for risk assessment of local regional recurrence still mainly rely on the visual detection of mucosal changes and histopathological assessment, which are unable to predict the tumor risk for most low grade premalignant lesions that develop at the former tumor site, consequently only leading to the identification of the recurrent tumor at a very advanced stage, *CIS* or SCC. Furthermore, this approach does not allow the clinician to detect recurrence in the absence of a clinically visible lesion (leukoplakia or erythroplakia). Therefore, there is great need to develop more reliable approaches that are better suited for accurate risk assessment of recurrence during follow-up.

I.7.2. Second primary tumors (SPTs)

Second primary tumors are a significant problem in treating oral SCC and have a negative impact on the prognosis of patients with oral cancer and their survival (Mashberg *et al.,* 2000). To define second primary tumor (SPT), most investigators currently use the criteria of Warren and Gates that were published in 1932: a second tumor is defined when both tumors are definitely malignant histologically and the second tumor is topographically distinct from the first tumor or chronologically distinct from the first tumor, and the probability of one being a metastasis of the other can be excluded. In theory, the histological evaluation for assessment of malignancies is easy and able to easily identify second primary tumors if the types of malignancies are different. For example, if the first 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 first tumor but a second primary tumor.

Unfortunately in practice, these criteria for differentiating recurrence and SPT lead to confusion. First, histologically identifying the nature of SOM can be difficult, with the types and grades of malignancies very similar, thus the histological possibility of a recurrence instead of SPT can not be ruled out in some cases. Secondly, there is no common agreement on the topographical criterion of differentiation of lesions, with no overall consensus on the appropriate distance between the index tumor and the second primary tumor that should be used as a cutoff value. Some investigators take 1.5 cm

(Scholes *et al.*, 1998), others 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). Thirdly, the criterion of chronological distinction for differentiation between recurrences and second primary tumors is also a matter of debate and confusion. Since the vast majority of SCC recurs within 5 years of treatment, a tumor developing from the same site beyond the 5-year span may be regarded as new tumor. There is, however, no hard evidence available to support this presumption (Funk *et al.*, 2002). Although more recent studies have shown that the chronological distinction time should be 3 years (Leong *et al.*, 1998; Shin *et al.*, 1996), some groups do not support chronological distinction as a criterion. One group has proposed that any subsequent SCC at the index tumor site or in 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).

I.7.3. Molecular biology of recurrences and SPTs

I.7.3.1. A genetically altered field as an explanation for recurrence and SPT

Recent molecular studies have shown that a tumor can be surrounded by a mucosal field consisting of genetically altered cells in a process termed "field cancerization". The term field cancerization was first used by Slaughter *et al* in 1953 to describe histologically altered epithelium surrounding tumor samples taken from the upper aerodigestive tract. Since then, it has been used to describe multiple patches of

pre-malignant disease and a higher-than-expected prevalence of multiple local recurrence and SPTs that were thought to be caused by the persistence of abnormal tissue after surgery. At the time of this study, there was limited molecular basis for this observation. However, many investigators have since then attempted to use molecular techniques to elucidate the mechanism that underlies the clinical phenomenon of field cancerization by assessing SOMs for clonality. This involves a determination of the base sequence of change in specific genes (such as *p53)* in the index and secondary lesions, or alternatively, a determination of similar LOH patterns in tumors and SOM. This idea of clonality has formed the basis by which researchers are beginning to categorize the development of multiple cancers.

Models are being proposed to explain the development of these fields of genetically altered cells and to determine the role that they play in carcinogenesis. One such model, developed by Braakhuis BJM (Braakhuis *et al.,* 2002) postulates that in the initial phase of carcinogenesis, a stem cell acquires genetic alterations and forms a 'patch', a clonal unit of altered daughter cells. These patches can be recognized on the basis of mutations in early molecular markers like LOH at 3p and 9p, and have been reported for head and neck, lung, skin and breast cancer. The conversion of a patch into an expanding field is the next logical and critical step in epithelial carcinogenesis. Additional genetic alterations are required for this step, and by virtue of its growth advantage, a proliferating field gradually displaces the normal mucosa. In the mucosa of the head and neck, as well as the esophagus, such fields have been detected with dimensions of > 7 cm in diameter, whereas they are usually not detected by routine

diagnostic techniques. The presence of a relatively large number of genetically altered stem cells in a field is a ticking time bomb, and as a result of the process of clonal divergence and selection, eventually the subclones evolve into one or more invasive tumors within a contiguous field of preneoplastic cells.

The concept of the expanding field in carcinogenesis has important clinical consequences. It is a well-known clinical experience that after surgical removal of a tumor, there is still a high risk of another tumor in the same anatomical area. An important clinical implication is that fields often remain after surgery of the primary tumor and may lead to new cancers, designated presently by clinicians as second primary tumor (SPT) or local recurrence, depending on the exact site and time interval. However, for cases in which treatment of the tumor has involved wide field excision, it seems logical to assume that a genetically altered field is the cause of the new cancer. The presence of a field with genetically altered cells appears to be a continuous risk factor for cancer. In addition, usually only a part of the fields are clinically visible as leukoplakia and erythroplakia, while some are clinically invisible. Therefore, there is an urgent need to identify the fields that carry the highest tumor risk for the early diagnosis of first primary tumor and prevention of local recurrence and SPTs. Molecular studies have begun to explore this problem, focusing on the identification of molecular markers for fields at risk. Microsatellite markers are among the more powerful of such approaches. As previously described in section I.5.1 and I.5.2 the presence of allelic loss at 3p and 9p has been shown in a retrospective study to be associated with an increased cancer risk (Patridge M

et al., 2000; Rosin MP *et al.,* 2000, 2002; Mao L *et al.,* 1996). However, there are few studies that utilize these markers to assess the tumor risk in a prospective manner.

I.7.3.2. Differentiation of recurrences and SPTs by "field cancerization"

Based on these advances in the molecular biology of field cancerization and molecular profiling of SOM (Scholes *et al.,* 1998; Chung *et al.,* 1993; Bedi *et al.,* 1996; Gasparotto *et al.,* 1995; Worsham *et al.,* 1995), a novel classification of a SOM after the primary carcinoma by comparing molecular patterns of first and second tumors has been proposed to differentiate the further tumors (Braakhuis *et al.,* 2002; Zhang *et al.,* 2001):

1. A recurrent tumor: all early molecular aberrations are similar to the ones in the index tumor, with a similar fingerprint for early molecular markers (e.g. LOH at 3p, 9p, and 17p) in recurrent and first tumors.
2. A "true SPT": the molecular profiles of tumors are different.

It is of clinical importance and significance to distinguish between a local recurrence and a SPT. When a new oral tumor develops in the same region where a small tumor has previously been excised and does so within, for example, 2 years, the type of treatment may depend on how this SOM is classified. In the case of local recurrence, many surgeons would be inclined to advise excision with postoperative radiotherapy, irrespective of the outcome of the histopathologic analysis of the surgical margins. On the other hand, when it is shown to be a SPT, unrelated to the first tumor, and the SPT

has been radically excised, there is often no need for further treatment. Another important issue with respect to the molecular classification of SOM is to provide a rational for novel forms of intervention (e.g., a gene-therapy-based approach), for example, in instances where conventional treatment would be felt to be ineffective. Finally, molecular techniques may aid in the analysis of surgical margins. The presence of altered clones at mucosal margins may be an indication for more aggressive therapy, including chemopreventive or radiotherapy, to treat altered clonal patches that have not been detected grossly by the pathologist and are beyond the initial scope of surgical excision.

I.7.3.3. LOH to determine clonality between lesions

Carcinogenesis is a dynamic process in which genetic alterations are acquired in a cumulative manner, often resulting in a continuum of benign, pre-malignant, and malignant neoplastic states. Through this process, successive waves of clonal outgrowth may occur as a cell population increases in aggressiveness and in its selective growth advantage. While it is widely accepted that the cells within this spectrum of progression are all genetically related, there is variability in the degree of relationship between cells that have diverged early on the pathway to malignancy. Therefore, the common theme in all of the molecular techniques that are utilized to pinpoint a clonal relationship is identification of early, shared genetic alterations that are unique to the lesions and not found elsewhere in normal tissue. As a result, these molecular patterns

form a type of DNA fingerprint and enable us to differentiate the relationship among the multiple oral lesions or tumors.

LOH defined by microsatellite analysis has recently been widely utilized to determine clonality between oral lesions. The molecular basis of this application is the head and neck cancer genetic progression model proposed in 1996 by Califano *et al.* (see section I.4.8) and the oral cancer genetic progression model established in our lab, which outline a temporal pathway for the accumulation of genetic alterations as defined by microsatellite loss. Markers that are altered early in the progression of HNSCC, such as LOH at 3p and 9p, could be utilized to identify and compare the genetic patterns of tumors. However, since no one marker is altered in all HNSCCs, a panel of markers needs to be used to determine the clonality. Microsatellite analysis has been demonstrated to be a very effective method of determining clonality, especially for detecting residual cancer cells whose outgrowth will give rise to the recurrence of cancer (van der Toorn, *et al.,* 2001; Slootweg *et al.,* 2002; Tabor *et al.,* 2004; van houten *et al.,* 2004). This thesis utilizes a group of LOH markers on 7 chromosomes to identify and compare the genetic similarity between the primary tumor and SOM.

I.8. The importance of post-therapy surveillance practice for OSCC

The theory of field cancerization suggests that there is an increased likelihood of concurrent or future cancers in patients with a history of oral cancer. Studies have

found that upwards of 50% of patients ultimately have a local or regional recurrence development (Vokes *et al.,* 1993) and 20-40% of patients will have a SPT occurrence (Haughey *et al.,* 1992). However, these further tumors (SOM) might be difficult to identify, requiring multiple examination modalities and/or frequent serial examinations. Timely discovery of treatment recurrence by an intensive follow-up regimen has been touted as a means to discover SOM as early as possible to permit effective treatment. Therefore, post-treatment surveillance has been widely accepted as a prominent component of oncologic practice, ostensibly to provide timely salvage intervention and to better coordinate recuperative support. However, follow-up of the patient with oral cancer is always questioned with regard to financial costs and effectiveness in the detection of early recurrences (Boysen *et al.,* 1992 and 1994; Wolfensberger *et al.,* 1988), although the post-treatment surveillance has been justified by the notion that early detection of recurrent or synchronous disease can improve salvage or, at the very least, direct supportive or rehabilitative interventions that can improve patient quality of life. The questions as to cost effectiveness revolve around issues of the difficulty in maintaining rigorous follow-up in the first 2 post-treatment years, with no more than 50% of patients keeping their appointments on a regular basis, and the inability to predict the malignant potential of lesions accurately, especially the "low-risk" lesions (leukoplakia), with current diagnostic tools. Therefore, improvement in the intensity of follow-up and the development of more accurate prognostic methods are key to the recognition of early SOM and further effective treatment.

This thesis utilizes a longitudinal study design to explore the role of intensive follow-up with a non-invasive molecular analysis as a means of more accurately predicting the risk of SOM after treatment. The data obtained provide a further support to the necessity of extensive post-treatment surveillance for such patients.

II. STATEMENT OF PROBLEM

Currently the prognosis for oral SCC remains one of the poorest among major human cancers, with a 50% five-year survival rate. This dismal prognosis is largely a result of late diagnosis of oral cancer and a high risk of these cancer patients developing SOM. Early identification and management of OPLs at high risk of progression into SOM are essential if we are to make a significant impact on this problem.

Need of non-invasive method of monitoring: Our current ability to identify OPLs at high-risk of developing into SOM is quite limited. Many of these OPLs are not clinically visible and patients with a history of oral cancer could have SOM development right under the watchful eyes of clinicians, despite intensive follow-up. Furthermore, even when OPLs are visible, they are not readily differentiated from reactive white and red lesions; hence biopsies may not be taken for the gold standard histology assessment. The situation is additionally complicated by the reluctance of clinicians to repeatedly biopsy such fragile sites (has a reduced capacity to regenerate), hence impeding the timely diagnosis of high-risk changes. Noninvasive approaches that could be used to identify the high-risk lesions are needed.

New markers are needed: Even when a clinical lesion is biopsied, the gold standard histology is not necessarily helpful if the degree of dysplasia is not pronounced. This is particularly a problem for lesions from the site of previous cancer and of aggressive treatment since reactive changes are common at these sites. Hence, it is important to

develop markers that could differentiate low-grade lesions at high-risk of developing into SOM from morphologically similar low-grade lesions that are at low-risk of developing into SOM.

Lack of data from prospective studies: Recent studies including those from this lab have shown that LOH patterns could predict the cancer risk of OPLs, and that exfoliative cells obtained non-invasively from oral mucosa could be used as a DNA source for LOH analysis. However, these studies are very preliminary in nature, all retrospective in design, and only from primary OPLs. Prospective studies, particularly those from patients with a history of oral cancer, are needed to validate the use of LOH as a tool to predict cancer risk of OPLs using both biopsy samples and exfoliative cells obtained non-invasively.

III. OBJECTIVES

1. To obtain samples from sites of previous oral cancer treated with a curative intention in patients currently being followed up in a large longitudinal study. These samples include both biopsy samples (if available) and exfoliative cell samples (if no biopsy was taken at the time).

2. To characterize the pattern of genetic change in the above samples by means of LOH analysis of 19 microsatellite markers for the following chromosomes 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*).

3. To compare the genetic pattern of samples from patients that later developed SOM with those that did not progress into SOM.

IV. HYPOTHESIS

Samples (both biopsies and exfoliative cells) from patients that later developed SOM will show an increased presence of high-risk LOH patterns as compared to samples from patients without development of SOM.

V. MATERIALS AND METHODS

V.1. Patients & Samples

V.1.1. Patients

The source of patients for this thesis is the Oral Cancer Prevention Longitudinal (OCPL) study funded by the National Institute of Dental Craniofacial Research (NIDCR). The OCPL study is described below.

V.1.1.1. OCPL study

This OCPL study is one of the first cohort studies of patients with oral lesions and is designed to systematically follow changes in clinical, pathological and molecular parameters over time. The study is an ongoing province-wide longitudinal study run jointly by the British Columbia Cancer Agency (BCCA), the University of British Columbia (UBC) and Simon Fraser University (SFU). The objective of the OCPL study is to identify molecular patterns that correlate with malignant transformation (for patients with oral premalignant lesions) or cancer recurrence (for cancer patients) and to use this information to develop a multi-faceted risk model with clinical application. Such studies have not been performed previously due to the difficulty in recruiting such patients for a longitudinal study.

V.1.1.2. The eligibility criteria for selecting patients in this study

Criteria including the following:

1. Aged 18 and over with a diagnosis of oral SCC, *CIS* or verrucous carcinoma (VC);
2. Treated with surgery, radiotherapy, or a combination of both, with an intent to cure;
3. Completed and signed informed consent for participating in the study;
4. Able to return to the Oral Oncology/Oral Dysplasia Clinic for regular follow-up;
5. Ability to communicate in English or have had a translator to help in communication;
6. At the time of the examination and scrape there was no recurrence or residual tumor present;
7. Availability of either a biopsy (when available) or a brushing sample (when biopsy unavailable) for LOH analysis
8. Availability of pathology reports and patient charts for review; and
9. Have been followed for at least 18 months, unless a recurrence had occurred prior to 18 months' follow-up.

An Institutional Review Board at each participating institution has approved the OCLP study. Patients with oral dysplasia or history of oral cancer who were referred to the Oral Oncology/Oral Dysplasia Clinic were given the information on the OCPL study and asked whether they were interested in participating in the study. All patients signed an

informed consent form at study entry. Patient participation is on a voluntary basis only and patients were told that they could terminate their participation in the study at any time. Patients involved in the study are given an identification number to ensure confidentiality outside the dental clinic. This identification number is then used to label all patient samples and to identify patients within the study database.

As of November 11 2004, the end-date for data acquisition for this thesis, the OCPL study had enrolled 202 patients aged 18 and over with a diagnosis of oral SCC, VC or *CIS*, and who were able to communicate and participate in regular follow-up. Of the 202 patients, 89 met the above eligibility criteria.

V.1.2. Collection of patient information

The following patient information was collected for the cases studied: demographic such as date of birth, age at the diagnosis of the index oral cancer, gender, and ethnicity, medical and family history, smoking habit, anatomical location of the lesions and follow-up time.

V.1.3. Collection of data on the index tumors

The following information was collected:

1. Tumor size and tumor stage (when possible) were determined ;

2. Tumor biopsy number was identified, and the biopsy report and histological slides were retrieved and reviewed. From these, the histological grading of the tumor (carcinoma *in situ*, well differentiated, moderately well differentiated, poorly differentiated SCC) was conducted;
3. Site of the tumor was determined from both the patient chart and thorough clinical examination;
4. Treatment of the tumor was reviewed, including type of the treatment, time of the treatment (for radiation, this include starting and ending time) and the name of the clinician for the treatment.

V.1.4. Follow-up of the site of previous tumor

This is a very important part of this study as the objective was to determine whether the LOH patterns of samples collected from the former cancer site would help clinicians identify high-risk areas for SOM. The clinical examination is conducted under the supervision of the attending Oral Medicine specialist.

When a new lesion was identified, it was recorded on a tracking sheet. Each lesion had a separate tracking sheet within a patient's file. The tracking sheet also records which procedures were done and which samples were collected on that date. These include lesion and non-lesion brushings, biopsies, blood draws, images, toluidine blue (TB) staining, autofluorescent visualization (FV), a saline wash and brushings of the buccal mucosa for exfoliated cells. Each lesion also has a form for any comments the clinician

wants to add that are beyond what is asked in the tracking sheets. All of this information is then uploaded into the OCPL database.

V.1.4.1. Taking samples during the follow-up

V.1.4.1.1. Biopsy

When a lesion was regarded as suspicious by the clinician, a biopsy was taken from the lesion, fixed in 10% formalin and submitted to the Oral Biopsy Service (OBS) for pathological assessment. The following information was recorded for the biopsy:

1) The site and size of the biopsy was marked on the tracking sheet.
2) The pathology requisition was completed with information on demographics, habits, history, clinical features of the lesion as well as TB staining and fluorescent visualization (FV, a new visual aid device being tested by the team) status of the lesion.
3) The nature of the biopsy, incisional (wedge or punch) vs. excisional.
4) The remaining clinical lesion size after biopsy (residual length and residual width).

V.1.4.1.2. Exfoliative cells (termed 'scrapes' or 'brushings')

Regardless of whether there were clinical lesions or whether a biopsy was to be taken, an exfoliative cell sample was always taken from each lesion, or site of the previous cancer

(if no lesion was present at the site) at each visit by using an Arcona cytology brush. A control exfoliative cells sample was taken from normal looking oral mucosa at a high-risk site, if available, at each visit. Each scrape was transferred to a cryovial containing Tris buffer and stored in liquid nitrogen for future digestion and DNA extraction.

V.1.5. Endpoint for follow-up

For this thesis, the main endpoint is the identification of SOM, including both recurrence and second primary tumor, occurring at the former tumor site. Patients that had a SOM development at the site of the previous cancer are defined as SOM cases. For those patients who did not develop a SOM (non-SOM cases), November 11, 2004, the cut-off date of this study, was used as the endpoint of follow-up. If the non-SOM patient had died before the cutoff date, the date of death was the endpoint.

V.1.6. Power of this study

Of the 89 patients used for my thesis, 25 developed a SOM at their previous cancer site, while 64 patients did not. The software, GPOWER, was used to calculate the power of this study and estimate the increased sample size of future study, in which the alpha value of 0.05 and the effect size of 0.5 were used. The analysis was two-sided.

V.2. Slide preparation

All biopsies assayed had to have sufficient epithelial and connective tissues for molecular analysis. For these cases, sections were cut from the archived paraffin blocks. One 5-micron-thick section was stained with hematoxylin and eosin (H&E) and coverslipped for reference. The actual samples for microdissection were 10 to 12 microns in thickness and each block had approximately 15 sections. They were also stained with H&E but left uncoverslipped. The H&E procedure is described below:

1. Slides were baked at 37°C overnight in an oven, then at 60° to 65°C for 1 hour, and left at room temperature to cool.
2. Samples were deparaffinized in two changes of xylene for 15 minutes.
3. Dehydration in gradient alcohols (100%, 95, 70% ethanol).
4. Hydrated by rinsing in tap water.
5. Slides were placed in Gill's Hematoxylin for 5 minutes then rinsed in tap water.
6. Blued with 1.5% (w/v) sodium bicarbonate then rinsed in water.
7. Slides were lightly counterstained with eosin, dehydrated, and cleared for coverslipping.
8. Thick sections to be dissected were stained by the above procedure without the dehydration step then air-dried.

V.3. Microdissection

Microdissection of the specimens was either performed or supervised by Dr. L. Zhang. Areas of hyperplasia, dysplasia and SCC were identified using H&E stained sections cut from formalin-fixed paraffin-embedded tissues. Epithelial cells in these areas were meticulously microdissected from adjacent non-squamous epithelium tissue or cells under an inverted microscope using a 23G needle. Genomic DNA from normal tissue was obtained by dissecting out the underlying stroma in these sections and used as control DNA for the case (Zhang *et al.,* 1997).

V.4. Sample digestion and DNA extraction

The brushing samples were thawed and centrifuged, and the pellets were resuspended in 300 µl of 50 mM Tris-HCL (pH 8.0) containing 1% SDS and proteinase K (0.5 mg/ml). The same enzyme solution was added to the microdissected tissue collected in a 1.5 ml eppendorf tube. All samples were incubated at 48°C for either 48 (scrapes) or 72 (microdissected tissue) hours. During incubation, samples were spiked with 10 or 20 µl of fresh 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, and washed with 70% ethanol. The samples were then re-suspended in LOTE, a low ionic strength Tris buffer and quantified fluorometrically (Rosin and Zhang, 1997).

V.5. DNA quantification

Fluorescence analysis with a Picogreen kit (Molecular Probes) was used to quantify DNA. This method used 2 standard curves. The low concentration standard curve was used for samples with 1 to 20 ng/µl, while the high concentration standard curve was used for concentrations between 10 and 400 ng/µl. Absorbance was read with a SLM 4800C spectrofluorometer (SLM Instruments Inc. Urbana, IL). The sample DNA concentration was then determined from one of the standard curves depending on its concentration, hence absorbance. A series of dilutions were done subsequently to adjust the concentration of DNA to 40 ng/µl with LOTE buffer (Rosin and Zhang, 1997).

V.6. Primer extension pre-amplification (PEP)

If the total amount of DNA was less than 200 ng, a procedure called PEP was used first to increase the DNA quantity. PEP involves amplification of multiple sites of the genome using random primers and low stringency conditions and hence increases the amount of total DNA for the microsatellite analysis. It was carried out in a 60 µl reaction volume containing 20 ng of the DNA sample, 900 mM of Tris-HCL of pH 8.3, 2 mM of dNTP where N is A, C, G and T, 400 µM of random 15-mers (Operon Technologies), and 1 µl of Taq DNA polymerase. Two drops of mineral oil were added

prior to the reaction. PEP using the automated thermal cycler (Omigene HBTR3CM, Hybaid limited) involved 1 cycle of pre-heat at 95°C for 2 minutes, followed by 50 cycles of: (1) denaturation at 92°C for 60 s, (2) annealing at 37°C for 2 min, and (3) polymerization at 55°C for 4 min (Rosin and Zhang, 1997).

V.7. End-Labeling

One more step prior to PCR was end-labelling of one member of the primer pair. The 50 μl reaction contained 38 μl of PCR standard water, 5 μl of 10 × buffer for T4 polynucleotide kinase (New England BioLabs), 1.2 μl of 100 × BSA, 100 ng of one of the primer pair, 3 μl of T4 polynucleotide kinase (New England BioLabs), and 2 μl of [γ-^{32}P] ATP (20 μCi, Amersham). The PCR reaction included 1 cycle at 37°C for 60 min run on the thermal cycler (Rosin and Zhang, 1997).

V.8. PCR amplification

DNA was analyzed for LOH by using microsatellite markers (Research Genetics, Huntsville, AL) that 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 oral premalignant and malignant lesions and were used for the previous publication (Zhang *et al.,* 1997).

PCR amplification using the thermal cycler was carried out in a 5 μl reaction volume containing 5 ng of genomic DNA, 1 ng of labelled primer, 10 ng of each unlabeled primer, 1.5 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 units of Taq DNA polymerase (GIBCO, BRL), PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH8.8), 6.7 mM magnesium chloride, 10 mM (-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide], and 2 drops of mineral oil. Amplification involved 1 cycle of pre-heat at 95C for 2 min; 40 cycles of 1) denaturation at 95 C° for 30s, 2) annealing at 50-60 C° (depending on the primer used) for 60s, and 3) polymerization at 70 C° for 60s; and 1 cycle of final polymerization at 70 C° for 5 min. The PCR products were then diluted 1:2 in loading buffer and separated on 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography. The films were then coded and scored for allelic imbalance (Zhang *et al.,* 1997).

V.9. Scoring of LOH

For informative cases (meaning two alleles are of different length and thus could be distinguished from one another by electrophoresis), LOH was scored if the signal intensity of the band was at least 50% less than its normal control counterpart from the

connective tissue DNA (Rosin and Zhang, 1997). All samples showing LOH were subjected to repeat analysis after a second independent amplification and re-scored whenever the quantity of DNA is sufficient.

V.10. Statistical analysis

Differences and associations between SOM and non-SOM groups were examined using either Fisher's exact test or Pearson's chi-square test for categorical variables (gender, smoking habit, stage, grade, and LOH) or unpaired t test for continuous variables (age and follow-up time). Time-to-SOM curves were estimated by Kaplan-Meier survival analysis. Relative risk (RRs) and the corresponding 95% confidence interval (CI) were determined using Cox regression analysis. All of the tests were two-sided. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed either with SPSS 12.0 or INSTAT 3.06 for Windows.

VI. RESULTS

The mean follow-up time for the 89 patients was 5.5 years (65 ± 38 months). Within this follow-up period, 28% (25/89) patients developed a SOM at the former cancer site (SOM group). The average time for the development of SOM was 35 (± 30) months, which is significantly shorter than the follow-up time for the 64 patients who did not develop SOM (non-SOM group) (62 ± 38; $P = 0.0009$).

VI.1. Clinicopathological features comparison of SOM and Non-SOM patients

Demographics and habit: Summary data for demographics and smoking habits of the 89 patients are listed in Table VI-1. Individual data is given in Appendix B. The mean age for the SOM group was 58 years (± 17) which is not significantly different from the non-SOM group (57 ± 15 years, P = 0.97). Although the gender distribution expressed as the percentage of male patients was different for the two groups, this difference was not statistically significant (60% in SOM vs. 30% in non-SOM, P = 0.098). For smoking habit, 68% of SOM cases reported tobacco use compared with 63% of non-SOM patients (P = 0.81).

Table VI-1. Demographics and habits

	All cases (%)	SOM (%)	Non-SOM (%)	*p*
Total	89 (100)	25 (100)	64 (100)	
Mean age (yr) ± SD	58 ± 15	58 ± 17	57 ± 15	*0.97*
Male sex---no. (%)	40/89 (45)	15/25 (60)	25/64 (39)	*0.098*
Tobacco use ever---no. (%)	57/88 (65)	17/25 (68)	40/63 (63)	*0.81*

Index tumors: The information on clinical parameters for the index tumors included staging, histology and treatment of the index carcinoma. These data are summarized in Table VI-2. When the tumor stages were examined, SOM patients had more *CIS* and early stage (1 & 2) tumors compared to non-SOM patient. The proportions of tumors in SOM patients with different stages were 46% *CIS*, 54% of stage 1/2 tumors, and 0% of stage 3/4 tumors. In comparison, the non-SOM patients had 19% *CIS*, 52% stage 1/2, and 12% stage 3/4 tumors ($P = 0.047$).

When the histological grade of the index tumors was examined, there was no significant difference in the tumor grading between SOM patients and non-SOM patients. Grade for the index tumors in SOM patients was 44% *CIS*, 52% well- and moderately-differentiated tumors, and 4% poorly-differentiated tumors. In comparison, the non-SOM patient group included 29% *CIS*, 57% well- or moderately-well differentiated tumors, and 14% poorly-differentiated tumors ($P = 0.22$).

No treatment difference was observed for the two groups: 80% of SOM cases and 67% of non-SOM cases received surgery only (*P* = 0.3) and 16% compared with 17% of SOM patients and non-SOM patient respectively, were treated with radiotherapy only (*P* = 1.0).

Because there was a significant difference in the stage of the tumor between SOM and non-SOM groups, a determination was also made of treatment among tumors of different stages, to ascertain whether they had been treated differently (Table VI-3). The results showed that the majority (93%) of stage 0 (*CIS*) tumors were treated surgically while the majority of stage 3 and 4 (83%) tumors were treated with radiation (42% with surgery, and 42% alone). Of those tumors treated surgically, *CIS* were also more likely to be treated with laser surgery compared to other surgically treated invasive SCC (37% vs. 15%, P = 0.073). These results would suggest that *CIS* and early invasive SCC (stage 1 and 2) may not have been treated aggressively enough, leaving residual tumor cells or severe dysplastic cells behind to later develop into a SOM after treatment.

Table VI-2. Clinicopathological features of target tumors

	All cases (%)	**SOM (%)**	**Non-SOM (%)**	***p***
Total	89 (100)	25 (100)	64 (100)	
Stage				
CIS (stage 0)	29/86 (34)	11/24 (46)	18/62 (29)	***0.047***
1 and 2	45/86 (52)	13/24 (54)	32/62 (52)	
3 and 4	12/86 (14)	0/24 (0)	12/62 (19)	
Histological grade				
CIS (stage 0)	29/88 (33)	11/25 (44)	18/63 (29)	*0.22*
Well or moderately well differentiated	49/88 (56)	13/25 (52)	36/63 (57)	
Poor differentiated	10/88 (11)	1/25 (4)	9/63 (14)	
Treatment of target carcinoma				
Surgery	62/88 (70)	20/25 (80)	42/63 (67)	*0.30*
Radiotherapy	15/88 (17)	4/25 (16)	11/63 (17)	*1.00*
Surgery & radiotherapy	11/88 (13)	1/25 (4)	10/63 (16)	*0.17*

Table VI-3. Treatment of index tumors of different stages

	All cases (#)	*CIS*	%	1+2	%	*P* 0 *vs.* 1+2	3+4	%	*P* 1+2 *vs.* 3+4
# of cases	86	29		45			12		
Surgery	62	27	93	31	69		2	17	
Radiotherapy	15	2	7	8	18	***0.0003***	5	42	***0.0046***
Surgery & radiotherapy	11	0	0	6	13		5	42	
Surgery only	62	27	93	31	69	***0.019***	2	17	***0.0021***
Radiation with or without surgery	26	2	7	14	31		10	83	

Index tumor site characteristics during follow-up: Information on clinical parameters at the index tumor site as collected during the follow-up period are shown in Table IV-4. At the date that the target samples were collected, 67% of the sites had a homologous clinical appearance in SOM patients compared with 68% of non-SOM patients ($P = 1.00$). Uptake of TB and the presence of OPLs at the former cancer site were both more frequent in SOM patients than non-SOM patients (TB: 45% *vs.* 12%; Presence of OPLs: 92% *vs.* 48%) with each of these comparisons being significantly different.

Table VI-4. Prior tumor site characteristics during follow-up

	All cases (%)	SOM (%)	Non-SOM (%)	*p*
Total	89 (100)	25 (100)	64 (100)	
Appearance				
Homologous	23/34 (68)	8/12 (67)	15/22 (68)	*1.00*
Nonhomologous	11/34 (32)	4/12 (33)	7/22 (32)	*1.00*
TB staining positive	10/54 (19)	5//11 (45)	5/43 (12)	***0.021***
Presence of lesions at former cancer sites	54/89 (61)	23/25 (92)	31/64 (48)	***< 0.0001***

VI.2. Microsatellite analysis of samples at previous cancer site during follow-up

VI.2.1. Microsatellite analysis of all samples (both biopsies and scrapes)

Table VI-5 and Figure 6 presents LOH data for all the cases and also compares LOH frequencies in SOM and non-SOM samples.

LOH was a frequent event in the OPLs that developed at former cancer sites. In total, 57% of the target samples taken from the previous cancer site had LOH on at least 1 region, 35% on multiple arms, 25% on 3p, 17% on 4q, 8% on 8p, 36% on 9p, 13% on 11q, 9% on 13q and 20% on 17p.

SOM samples showed consistently higher frequencies of LOH than those of non-SOM (Figure. 6). When individual arms were analyzed, a significantly higher frequency of LOH was noted in SOM samples for the following chromosome arms: 3p (48% *vs.* 16% in non-SOM; $P = 0.0026$), 4q (32% *vs.* 11%; $P = 0.045$), 9p (72% *vs.* 22%; $P < 0.0001$) and 17p (61% *vs.* 11%, $P = 0.0011$). SOM lesions also showed significantly increased high-risk LOH patterns: multiple LOH (72% vs. 20% in non-SOM; $P < 0.0001$); LOH at 3p and/or 9p plus others chromosomes (64% vs. 19%; $P < 0.0001$); and LOH at 3p &/or 9p (84% vs. 31%; $P < 0.0001$) (Figure 6). Figure 7 presents an example of comparison of LOH patterns between a SOM case and a non-SOM case.

Table VI-5. LOH and SOM

	All (%) (n = 89)	**SOM (%) (n = 25)**	**Non-SOM (%) (n = 64)**	***p***
No. with LOH [a]	51/89 (57)	22/25 (88)	29/64 (45)	***0.0003***
> 1 arm lost	31/89 (35)	18/25 (72)	13/64 (20)	***< 0.0001***
> 2 arm lost	21/89 (24)	14/25 (56)	7/64 (11)	***< 0.0001***
LOH on 3p	22/89 (25)	12/25 (48)	10/64 (16)	***0.0026***
LOH on 9p	32/89 (36)	18/25 (72)	14/64 (22)	***< 0.0001***
LOH on 17p	18/89 (20)	11/25 (61)	7/64 (11)	***0.0011***
LOH on 4q	14/83 (17)	7/22 (32)	7/61 (11)	***0.045***
LOH on 8p	7/87 (8)	4/24 (17)	3/63 (5)	*0.088*
LOH 11q	11/88 (13)	5/25 (20)	6/63 (10)	*0.28*
LOH on 13q	7/81 (9)	3/21 (14)	4/60 (7)	*0.37*
LOH on 3p and/or 9p only	13/89 (15)	5/25(20)	8/64 (13)	*0.50*
LOH on 3p and/or 9p LOH (plus LOH at any other arms)	28/89 (31)	16/25(64)	12/64 (19)	***< 0.0001***
All cases with LOH on 3p and/or 9p	41/89 (46)	21/25 (84)	20/64 (31)	***< 0.0001***

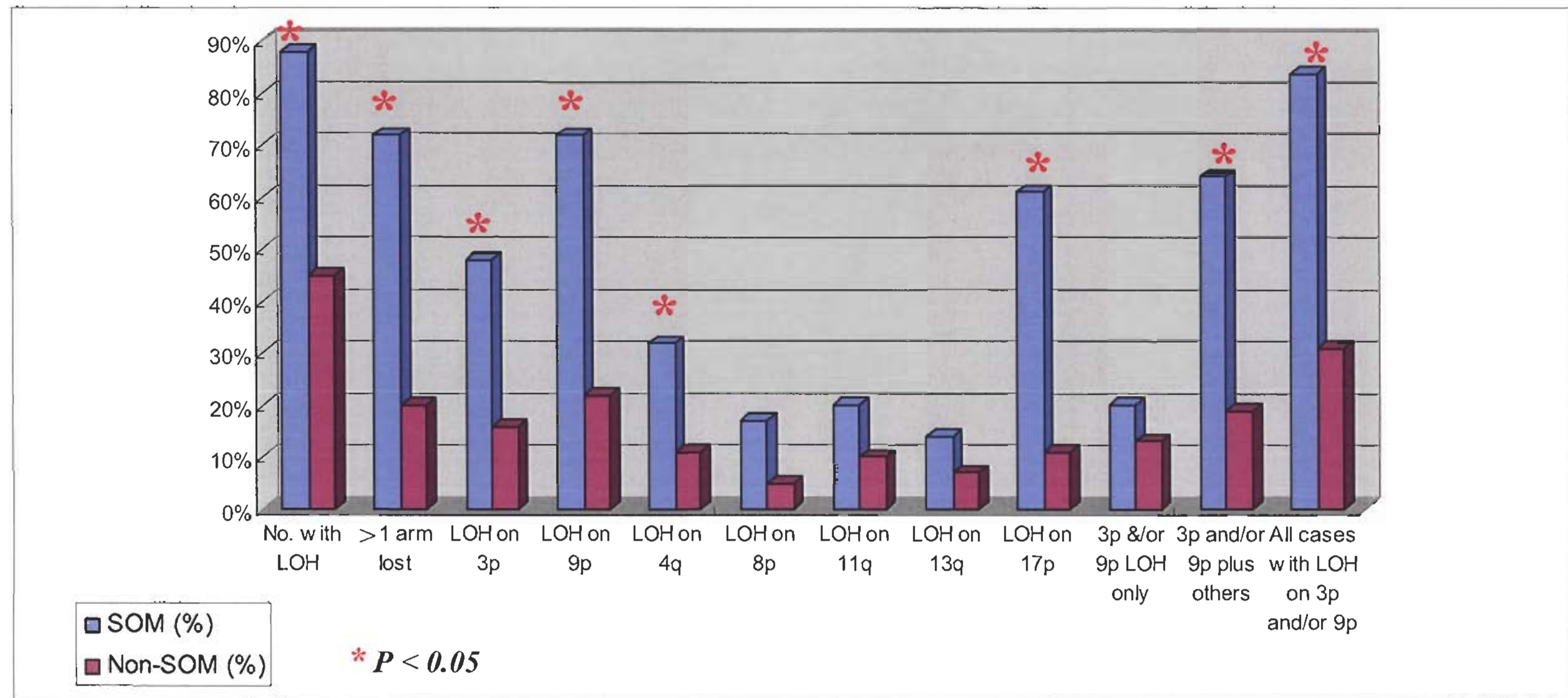


Figure 6. Allelic loss in samples from patients with SOM and without SOM

Proportion of SOM and non-SOM patients showing indicated LOH patterns. SOM patients showed a significantly higher frequency of LOH than non-SOM patients for four chromosome arms: 3p (48% *vs*. 16% in non-SOM; $P = 0.0026$), 4q (32% *vs*. 11%; $P = 0.045$), 9p (72% *vs*. 22%; $P < 0.0001$) and 17p (61% *vs*. 11%, $P = 0.0011$), but not for 8p, 11q, and 13q. Also, SOM lesions showed significantly increased high-risk LOH patterns: multiple LOH (72% vs. 20% in non-SOM; $P < 0.0001$); LOH at 3p and/or 9p plus any of other chromosome arms (64% vs. 19%; $P < 0.0001$); and LOH at 3p &/or 9p irrespective of alteration at any other arm (84% vs. 31%; $P < 0.0001$).

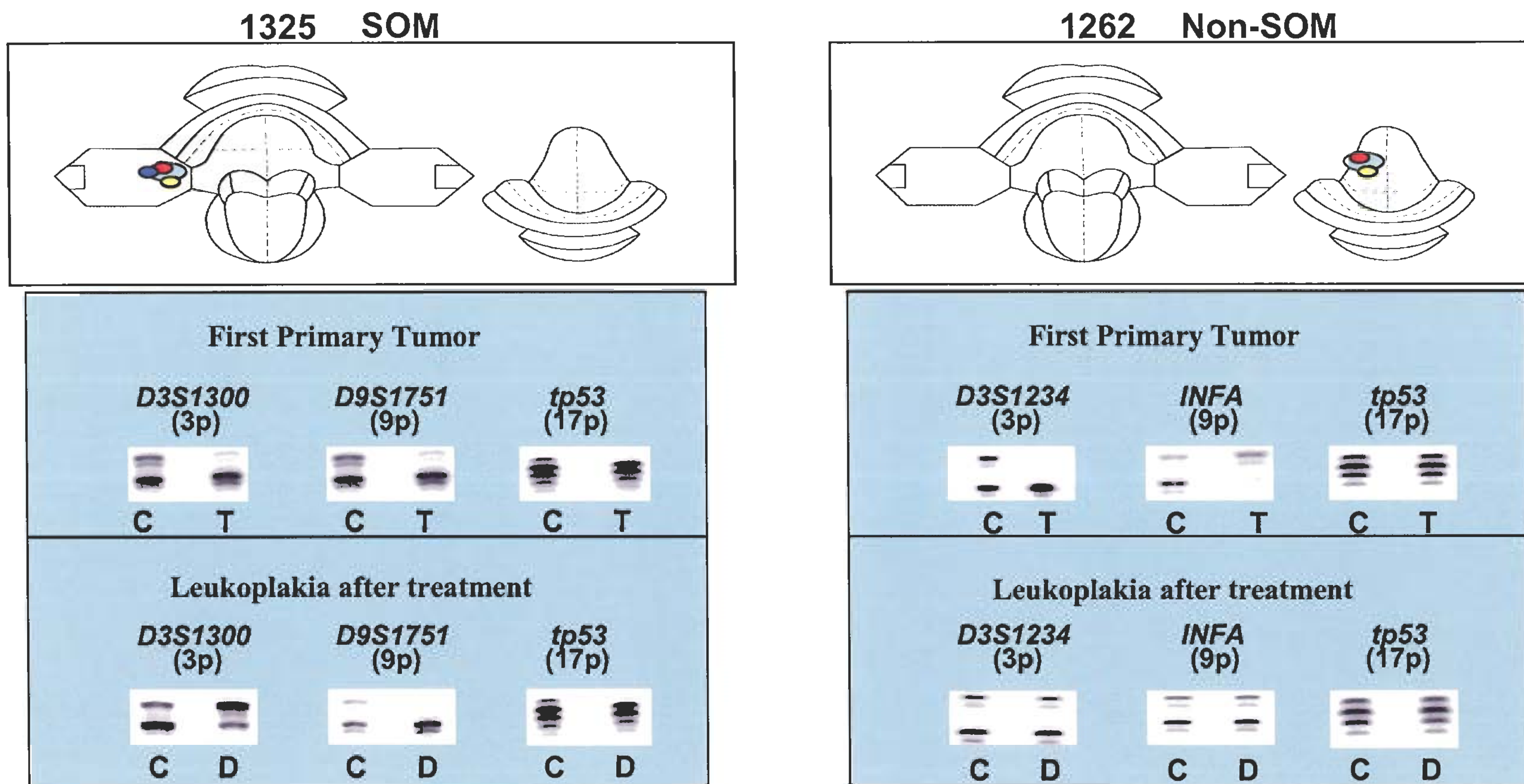


Figure 7. LOH pattern comparison between the previous cancer and the target sample

Examples of LOH patterns in primary tumor and recurrent leukoplakia for 2 cases, an SOM and a non-SOM patient. Patient 1325 had a SOM development at the former cancer site while patient 1262 did not. The LOH patterns for 3p, 9p, and 17p were compared between the index tumor and the subsequent sample taken from the previous cancer site. LOH was absent in the leukoplakia that did not develop into SOM, but present in the cases (1325) that did.

VI.2.2. Microsatellite analysis of brushing samples

One of the objectives of the thesis was to validate the value of the brushing samples taken noninvasively as a DNA source for LOH analysis. Table VI-6 presents all the brushing LOH data and compares the LOH results from brushings in SOM group and non-SOM group.

The results showed that brushing samples could serve as a DNA source for LOH analysis. Of the 57 brushing samples used in this thesis, 51% had LOH on at least 1 region, 43% on 3p, 29% on 4q, 7% on 8p, 46% on 9p, 32% on 11q, 14% on 13q, and 18% on 17p. The percentage of patients with 3p and/or 9p LOH was significantly elevated in the SOM group (56% *vs.* 10%, P = 0.0074), similar to the overall findings when all samples (scrapes and biopsies) were used in the comparison.

Table VI-6. LOH in scrape samples

	All (%) (n = 57)	SOM (%) (n = 9)	Non-SOM (%) (n = 48)	*p*
No. with LOH [a]	29/57 (51)	6/9 (67)	23/48(82)	*0.47*
> 1 arm lost	13/28 (46)	3/9 (33)	10/48 (21)	*0.41*
> 2 arm lost	7/28 (25)	2/9 (22)	5/48 (10)	*0.30*
LOH on 3p	12/28 (43)	4/9 (44)	8/48 (17)	*0.082*
LOH on 9p	13/28 (46)	3/9 (33)	10/48 (21)	*0.41*
LOH on 17p	5/28 (18)	1/9 (11)	4/48 (8)	*1*
LOH on 4q	8/28 (29)	1/8 (13)	7/40 (18)	*1*
LOH on 8p	2/28 (7)	0/8 (0)	2/43 (5)	*1*
LOH 11q	9/28 (32)	3/8(38)	6/42 (14)	*0.14*
LOH on 13q	4/28 (14)	1/6 (17)	3/42 (7)	*0.42*
LOH on 3p and/or 9p only	8/28 (29)	2/9 (22)	6/48 (13)	*0.60*
LOH on 3p and/or 9p LOH (plus LOH at any other arms)	13/28 (46)	3/9 (33)	10/48 (21)	*0.41*
All cases with LOH on 3p and/or 9p	21/28 (75)	5/9 (56)	5/48 (10)	***0.0054***

VI.3. Microsatellite analysis of samples at previous cancer sites during follow-up for tumors of different stages

Since there was a significantly higher number of late stage tumors in the non-SOM group, a separate comparison of LOH results was also made for tumors of the same stage to see if LOH remained a cancer risk marker (Table VI-7 & 8). For the patients with tumors of stage 0, significantly higher frequencies of LOH were observed in the SOM group than the non-SOM group: LOH on at least 3 regions: 55% *vs.* 11% (P = 0.028); 3p LOH: 64% *vs.* 17% (P = 0.017); 9p LOH: 64% *vs.* 17% (P = 0.017); 17p LOH: 55% *vs.* 17% (P = 0.048); LOH on 3p and/or 9p plus other chromosomes: 73% *vs.* 17% (P = 0.0051); and all cases with 3p and/or 9p LOH: 82% *vs.* 28% (P = 0.0078). Similarly, elevated LOH patterns were noted in the SOM group in tumors of stage 1 and 2: LOH on at least 1 region: 85% vs. 47% (P = 0.024); multiple LOH: 69% vs. 22% (P = 0.0051); 9p LOH: 77% *vs.* 25% (P = 0.0022); 17p LOH: 31% *vs.* 3% (P = 0.020); LOH on 4q: 60% vs. 13% (P = 0.0074); LOH on 3p and/or 9p plus other chromosomes: 54% *vs.* 19% (P = 0.03); and all cases with 3p and/or 9p LOH: 85% *vs.* 38% (P = 0.0074). These results were similar to the analyses using all index tumors of different stages, supporting the use of LOH as predictor for SOM risk independent of the stage of the prior tumor.

Table VI-7. LOH in tumors of stage 0

Stage 0	**All (%) (n = 89)**	**SOM (%) (n = 25)**	**Non-SOM (%) (n = 64)**	***p***
No. with LOH [a]	20/29(69)	10/11(91)	10/18(56)	*0.096*
>1 arm lost	11/29(38)	8/11(73)	3/18(17)	*0.15*
>2 arm lost	8/29(28)	6/11(55)	2/18(11)	***0.028***
LOH on 3p	10/29(34)	7/11(64)	3/18(17)	***0.017***
LOH on 9p	10/29(34)	7/11(64)	3/18(17)	***0.017***
LOH on 17p	9/29(31)	6/11(55)	3/18(17)	***0.048***
LOH on 4q	3/22(14)	1/9(11)	2/13(15)	*1*
LOH on 8p	4/27(15)	1/11(9)	3/16(19)	*0.62*
LOH 11q	4/25(16)	3/11(27)	1/14(7)	*0.29*
LOH on 13q	2/24(8)	1/9(11)	1/15(7)	*1*
LOH on 3p and/or 9p only	3/29(10)	1/11(9)	2/18(11)	*1*
LOH on 3p and/or 9p LOH (plus LOH at any other arms)	11/29(38)	8/11(73)	3/18(17)	***0.0051***
All cases with LOH on 3p and/or 9p	14/29(48)	9/11(82)	5/18(28)	***0.0078***

Table VI-8. LOH in tumors of stage 1+2

Stage 1+2	All (%) (n = 89)	SOM (%) (n = 25)	Non-SOM (%) (n = 64)	*p*
No. with LOH [a]	26/45(58)	11/13(85)	15/32(47)	***0.024***
>1 arm lost	16/45(36)	9/13(69)	7/32(22)	***0.0051***
>2 arm lost	10/45(22)	7/13(54)	3/32(9)	***0.0029***
LOH on 3p	11/45(24)	5/13(38)	6/32(19)	*0.25*
LOH on 9p	18/45(40)	10/13(77)	8/32(25)	***0.0022***
LOH on 17p	5/45(11)	4/13(31)	1/32(3)	***0.020***
LOH on 4q	10/40(25)	6/10(60)	4/30(13)	***0.0074***
LOH on 8p	2/42(5)	2/12(17)	0/30(0)	*0.077*
LOH 11q	7/43(16)	2/12(17)	5/31(16)	*1*
LOH on 13q	4/42(10)	2/11(18)	2/31(6)	*0.28*
LOH on 3p and/or 9p only	10/45(25)	4/13(31)	6/32(19)	*0.44*
LOH on 3p and/or 9p LOH (plus LOH at any other arms)	13/45(29)	7/13(54)	6/32(19)	***0.03***
All cases with LOH on 3p and/or 9p	23/45(51)	11/13(85)	12/32(38)	***0.0074***

VI.4. Clonal relationship between prior tumors and target samples defined by the comparison of LOH patterns: same clone origin or not?

Table VI-9 presents a comparison of data in tumor and follow-up samples, to determine the clonal relationship between the index tumor and the target lesion. The comparison of the genetic changes between the index tumor and the target lesion was made in three steps:

Step 1: comparison of only 3p and 9p markers (up to 7 microsatellite markers were used).

LOH at these two chromosomes is the earliest event.

Step 2: comparison of 3p, 9p, 17p, and 4q markers (up to 12 primers used).

Step 3: comparison of 8p, 11q, and 13q (these are late events), in addition to 3p, 9p, 17p and 4q (up to 19 primers used).

Three results are categorized in the following fashion in the table below:

1. Same: when data for all of the microsatellite markers between the index tumor and the target lesions were the same (e.g., both retention, or both showing loss of the same alleles for each locus examined). We allowed the data for the 2 samples to have an additional loss for 1 of the markers examined to allow for additional changes occurring during the SOM development, that could occur even when the later lesion was clonally related to the index tumor. There is a precedent for doing this in the literature (Zhang *et al.,* 2001).
2. Maybe the same: same as above but accept one difference, that is, the index tumor had a loss but target sample showed retention. This is possible as a result of treatment effect, or dominant clone outgrowth.
3. Different: When there are two differences as described above, or whenever the losses in the index tumor and the target sample were different for a particular marker, e.g., upper allele loss at 9pINFA for the index tumor but lower allele for the target sample.

The LOH pattern comparison between index tumor and target lesions is summarized in table VI-9. For the 3p and 9p marker comparison, 50% of target lesions in SOM patients had the same LOH patterns as the index tumors and 13% fell into the category of "maybe same". In comparison, only 16% of non-SOM cases shared the same LOH fingerprint with the prior tumors (P = 0.0084). In the step 2 comparison with 17p and 4q markers added, 25% "same" and 29% "maybe same" in SOM cases compared to 6% "same" and 16% "maybe same" in non-SOM cases (P = 0.013). In step 3, when all 19 markers were used, a significant difference in number of percent of "same" and "maybe same " LOH patterns between SOM and non-SOM group was still noted (P = 0.021). All these data strongly suggest that either leftover cancer cells or clonally related premalignant cells at the former cancer site make a significant contribution to the high frequent tumor recurrence after treatment (Table IV-9 & Figure 8).

Table VI-9. Clonal relationship between index tumors and target samples

	Non-SOM	%	SOM	%	*p*
# of cases	50		24		
3p and 9p					
Same	8	16%	12	50%	***0.0084***
Maybe same	9	18%	3	13%	
Different	33	66%	9	38%	
3p, 9p, 4q and 17p					
Same	3	6%	6	25%	***0.013***
Maybe same	8	16%	7	29%	
Different	39	78%	11	46%	
All					
Same	1	2%	5	21%	***0.021***
Maybe same	5	10%	2	8%	
Different	44	88%	17	71%	

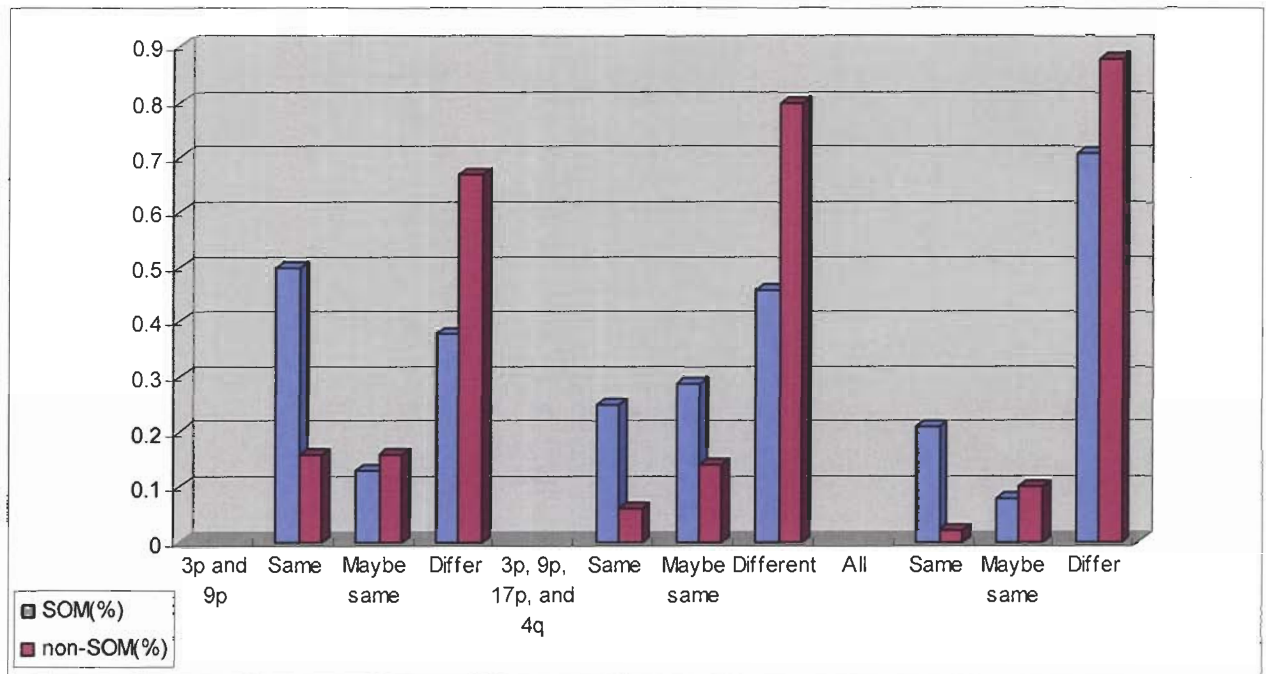


Figure 8. Same LOH pattern showed in both prior cancers and target samples
LOH profile was compared between index tumor and target lesion for 3p and 9p; 3p, 9p, 17p, and 4q; 3p, 9p, 17p, 4q, 8p, 11q, and 13q respectively. For each comparison, a significantly higher percent of lesions that later progressed into SOM were found to have identical or near identical LOH profiles to the index tumors in SOM compared to non-SOM cases.

VI.5. Time to outcome for different LOH patterns

Specific LOH patterns in the samples taken from the previous cancer site were examined for association with SOM development by using the Cox proportional hazard regression method, and "time-to-SOM" curves were estimated by the Kaplan-Meier method which is a statistical survival analysis.

Data for each individual arm were analyzed. Time-to-development of SOM was significantly decreased for cases with LOH at 3p, 4q, 9p, and 17p (Figure 9, *A, B, D, G*; $P < 0.05$), but not significant for 8p, 11q, and 13q (Fig 9, *C, E, F*; $P > 0.05$).

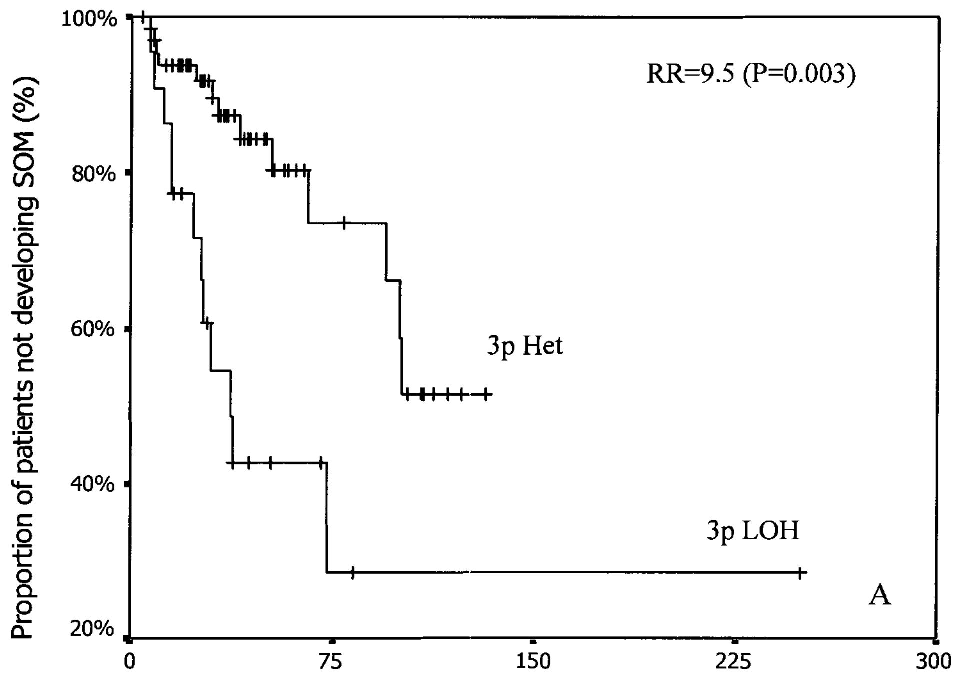
Since previous study has shown loss of 3p &/or 9p markedly increased cancer recurrence risk, the study also compared the following combinations: LOH at 3p &/or 9p only; LOH at 3p &/or 9p plus LOH at any of 4q, 8p, 11q, 13q, or 17p; LOH at 3p &/or 9p. A highly significant difference was observed for the LOH pattern of 3p and/or 9p plus others ($P < 0.0001$), or simply anytime there was LOH at 3p &/or 9p ($P < 0.0001$).

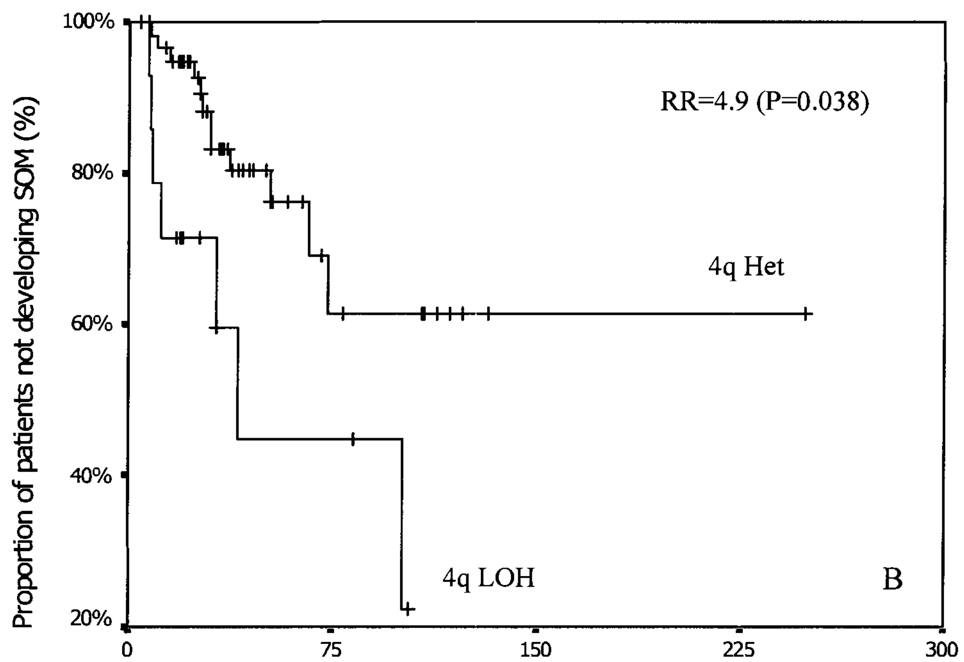
An assessment of relative risk (RR) of SOM development was performed for each of the above LOH patterns and combinations (Table VI-10). The results showed that the RRs were significantly higher for LOH at the following chromosome arm: 3p Het *vs.* 3p LOH (9.5- fold, 95% CI: 1.8-14.0), 4q Het *vs.* 4q LOH (4.9-fold 95% CI: 1.2-13.6), 9p Het *vs.* 9p LOH (19.4-fold 95% CI: 9.2-26.4) and 17p Het *vs.* 17p LOH (11.1-fold 95% CI:

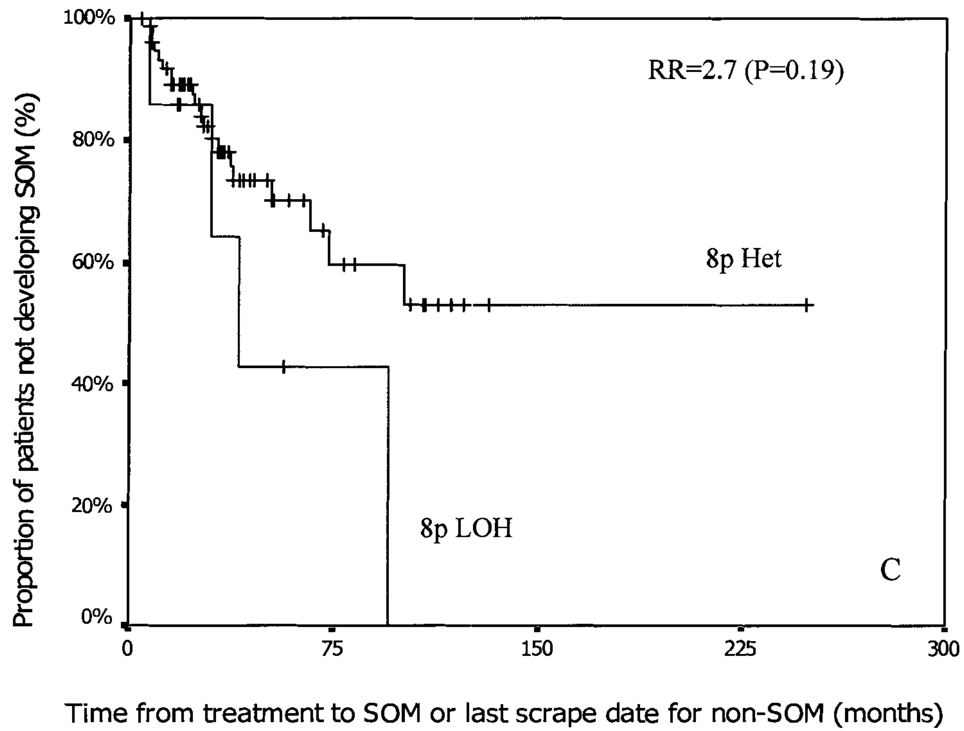
2.1-19.5). For the combined pattern, a marked increase in RR was noted when LOH at 3p &/or 9p were combined. There was a strikingly increased RR for cases with 3p and/or 9p LOH plus others (21.8-fold, 95% CI: 4.1-52.1), or just LOH at 3p &/or 9p (21.4-fold, 95% CI: 3.5-38.1).

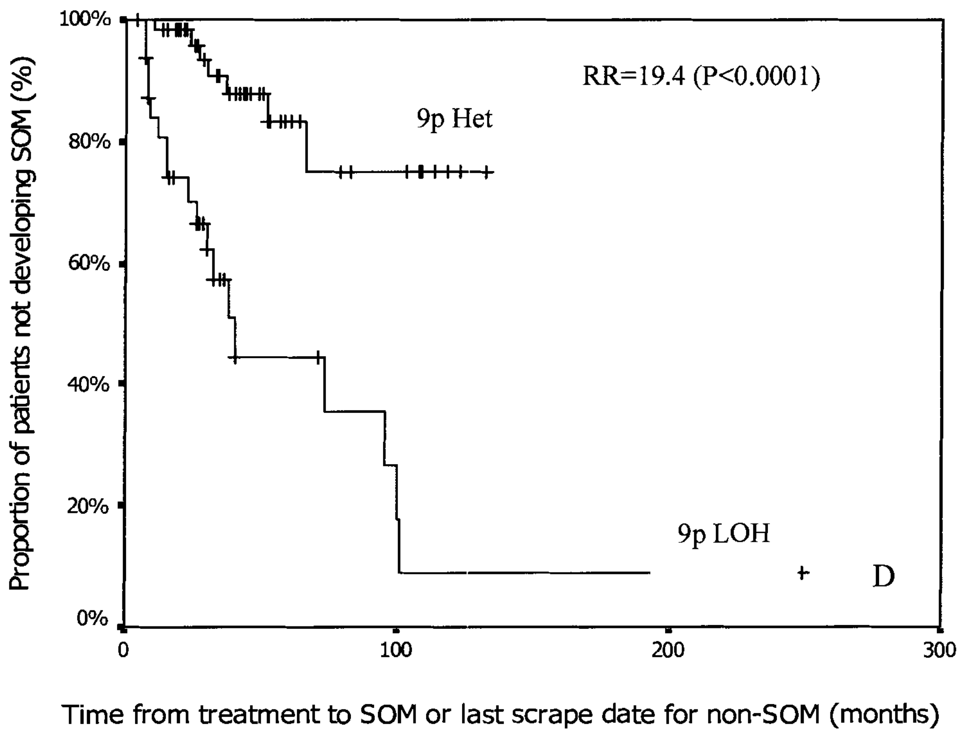
Table VI-10. RRs (95% CI) of samples from prior cancer site developing into a SOM

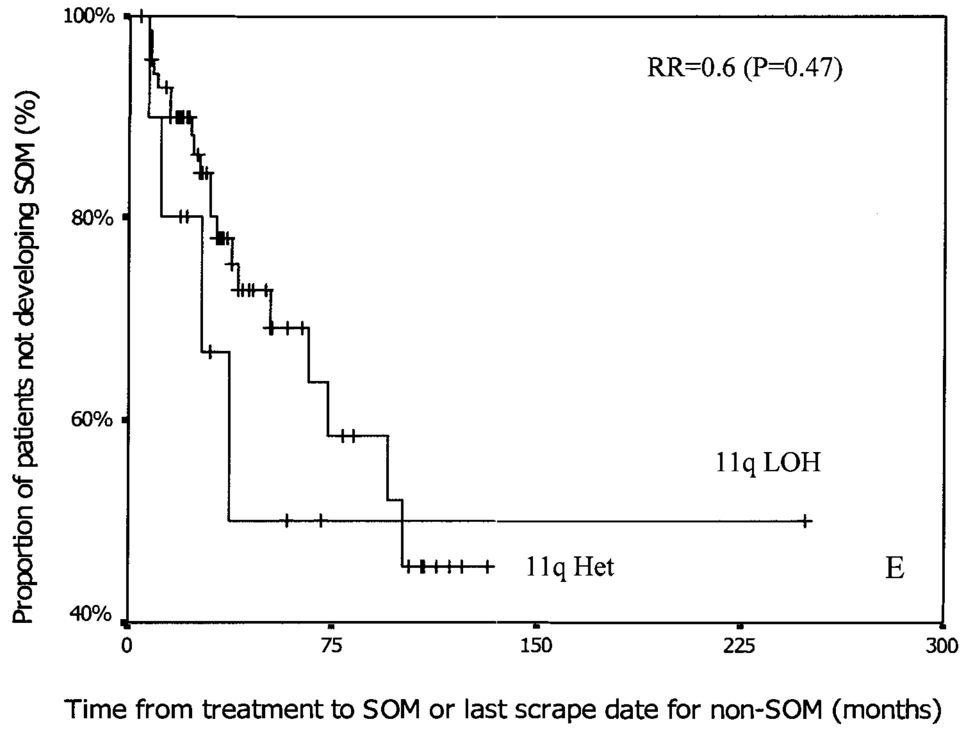
LOH pattern	**RR (95% CI)**	***p***
3p Het vs. LOH	9.5 (1.8-14.0)	*0.003*
9p Het vs. LOH	19.4 (9.2-26.4)	*< 0.0001*
4q Het vs. LOH	4.9 (1.2-13.6)	*0.038*
8p Het vs. LOH	2.7 (0.8-18.2)	*0.19*
11q Het vs. LOH	0.6 (0.4-6.7)	*0.47*
13q Het vs. LOH	1 (0.5-11.0)	*0.38*
17p Het vs. LOH	11.1 (2.1-19.5)	*0.001*
3p and/or 9p Het vs. LOH		
3p and/or 9p LOH only (no other arms)	4.9 (1.3-31.1)	*0.033*
3p and/or 9p LOH (plus LOH at any other arms)	21.8 (4.1-52.1)	*< 0.0001*
All cases with 3p and/or 9p LOH	21.4 (3.5-38.1)	*< 0.0001*


100%
80%
60%
40%
20%
0
75
150
225
300
RR=9.5 (P=0.003)
3p Het
3p LOH
A
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)

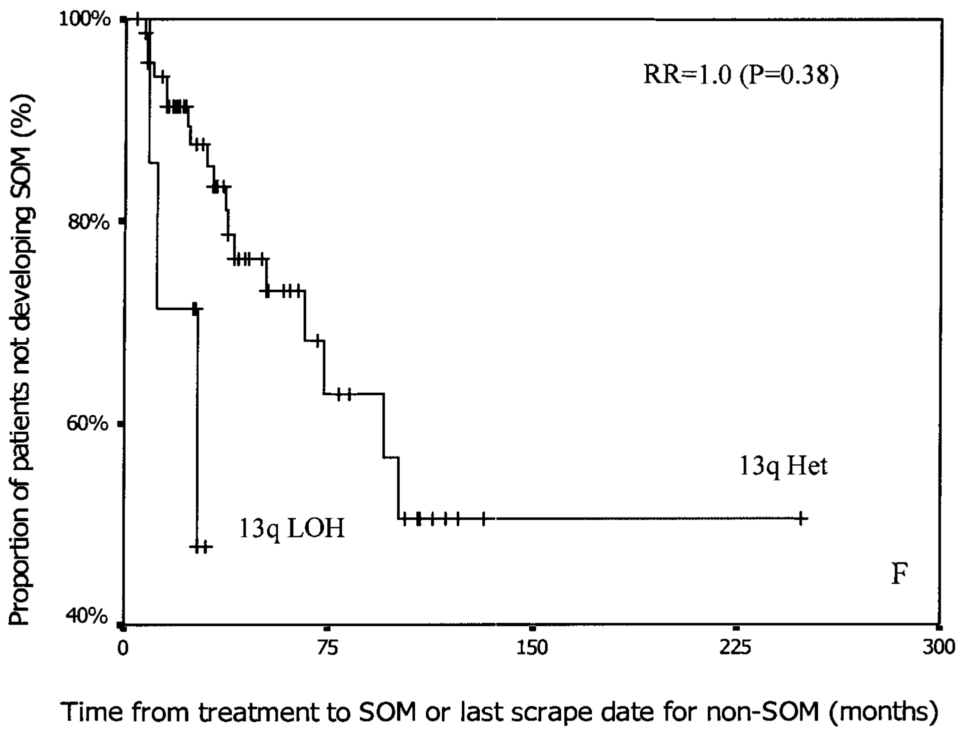

100%
80%
60%
40%
20%
0
75
150
225
300
RR=4.9 (P=0.038)
4q Het
4q LOH
B
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)


RR=2.7 (P=0.19)
8p Het
8p LOH
C
100%
80%
60%
40%
20%
0%
0
75
150
225
300
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)

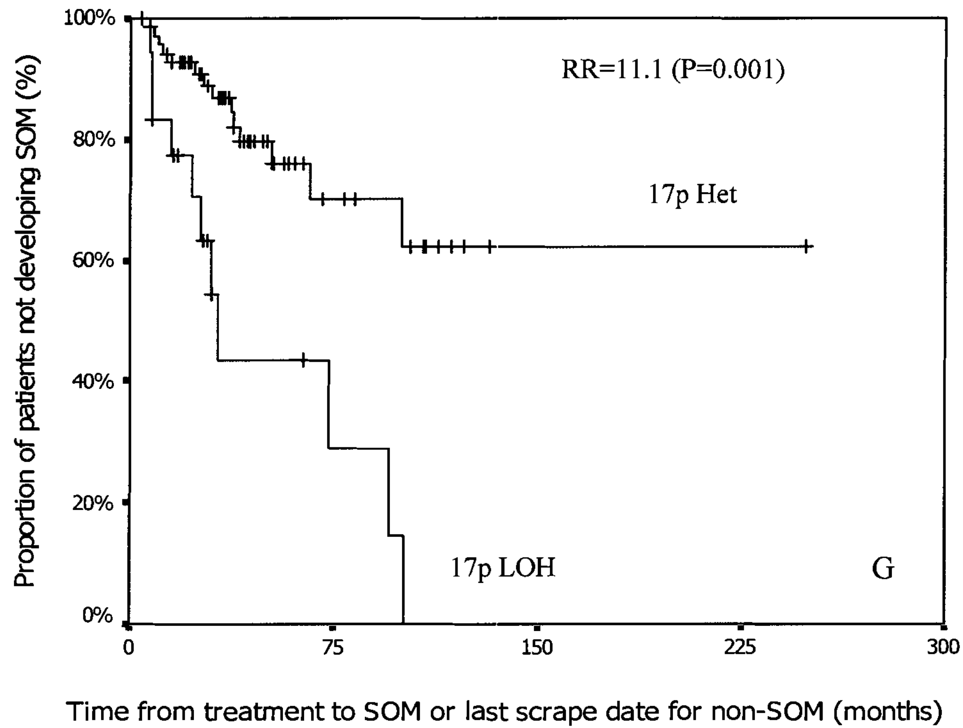

RR=19.4 (P<0.0001)
9p Het
9p LOH
D
100%
80%
60%
40%
20%
0%
0
100
200
300
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)

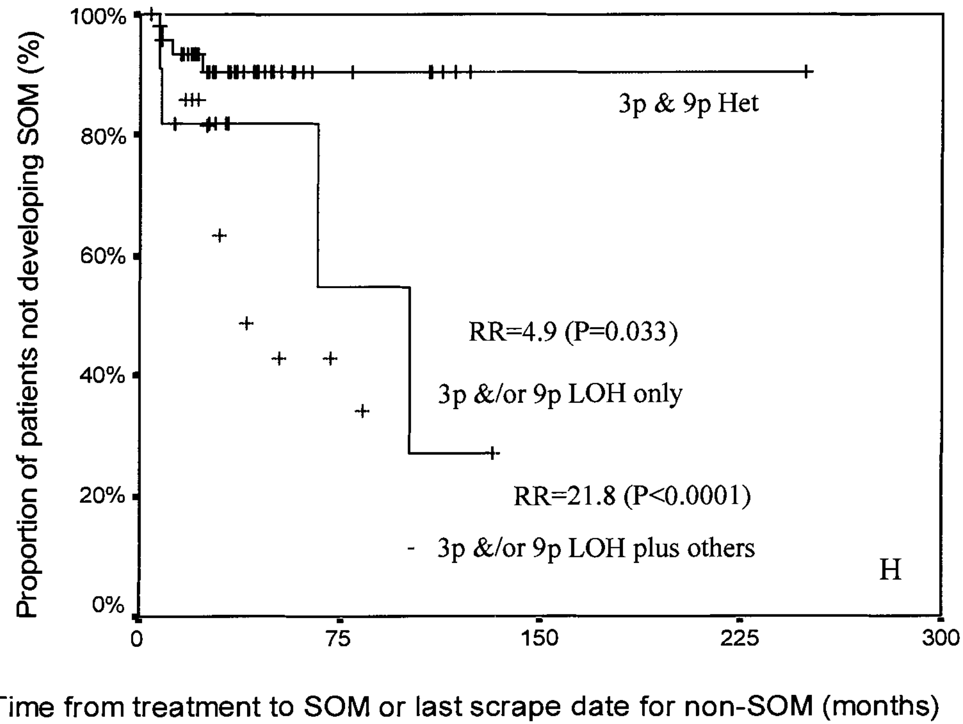


RR=0.6 (P=0.47)
11q LOH
11q Het
E
100%
80%
60%
40%
0
75
150
225
300
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)



RR=1.0 (P=0.38)
13q Het
13q LOH
F
100%
80%
60%
40%
0
75
150
225
300
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)


100%
80%
60%
40%
20%
0%
0
75
150
225
300
RR=11.1 (P=0.001)
17p Het
17p LOH
G
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)


100%
80%
60%
40%
20%
0%
0
75
150
225
300
3p & 9p Het
RR=4.9 (P=0.033)
3p &/or 9p LOH only
RR=21.8 (P<0.0001)
- 3p &/or 9p LOH plus others
H
Proportion of patients not developing SOM (%)
Time from treatment to SOM or last scrape date for non-SOM (months)

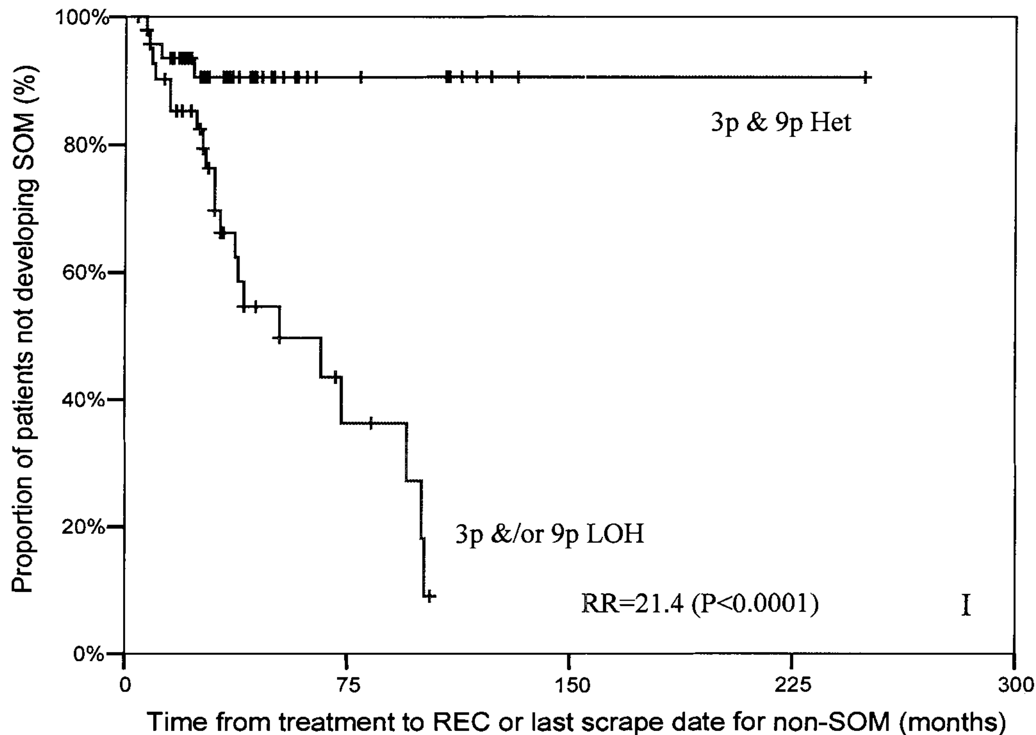


Figure 9. Probability of development of a SOM at former cancer sites

Figure 9 is the probability of development of a SOM at former cancer sites according to LOH pattern. *A*, progression as a function of LOH at 3p (RR=9.5, P=0.003); *B*, progression as a function of LOH at 4q (RR=4.9, P=0.038); *C*, progression as a function of LOH at 8p (RR=2.7, P=0.19); *D*, progression as a function of LOH at 9p (RR=19.4, P<0.0001); *E*, progression as a function of LOH at 11q (RR=0.6, P=0.047); *F*, progression as a function of LOH at 13q (RR=1.0, P=0.38); *G*, progression as a function of LOH at 17p (RR=11.1, P=0.001); *H*, progression as a function of LOH at 3p and/or 9p when this loss occurred in the absence or presence of LOH at any other arm (RR=4.9, P=0.033; RR=21.8, P<0.0001); *I*, progression as a function of LOH at 3p and/or 9p (RR=21.4, P<0.0001).

VII. DISCUSSION

VII.1. Clinical pathological features and SOM

Individuals with prior history of oral SCC have significant risk of SOMs, including both recurrences and SPT. Early diagnosis and management of oral lesions at premalignant stages before they progress into SOMs would significantly improve the current dismal prognosis of oral cancer. However our current abilities to identify these lesions are very limited. For example, studies have shown that a number of characteristics of the index tumor could have predictive value for the frequency of recurrence. These include an advanced stage of the disease or poor histological differentiation, each of which has been associated with a higher probability of SOM. However, on an individual basis, such information adds little information that would assist a clinician in the early identification of a developing SOM.

We also have a number of clinicopathological risk factors that could be used to watch for high risk changes at the site of prior cancer, a very high-risk region. Features such as the size and appearance of the lesions, and presence and degree of dysplasia of the lesion could alert clinicians to the possibilities of high-risk lesions progressing into SOM. Recent studies also showed that TB could help the clinicians to identify early high-risk oral lesions (Mashberg A *et al.,* 1995; Martin IC *et al.,* 1998; Guo Z *et al.,* 2001; Epstein JB *et al.,* 2003). However, little or no research has been done to see whether the

clinicopathological risk factors of primary OPLs apply to OPLs at sites of previous oral SCC. One of our research team's goals is to rigorously collect clinicopathological changes of the sites of prior cancer during the longitudinal follow-up. A clinician's ability to predict which lesion is at a high-risk for a SOM prior to a second cancer diagnosis would be very advantageous. With this information treatment can be performed with a minimal amount of morbidity and with less emotional distress to the patient.

The collection of these data involved multiple people in the research team including clinicians, a hygienist, a dental assistant, data managers, and pathologists. During the study period, 25 of the 89 patients developed a SOM at the previously treated cancer site, 44% within 2 years, 32% within 3 years, and 24% after 3 years. The team investigated the relationship between SOM and the following parameters: demographic features, tobacco habits, target tumor information, and clinical features of post-treatment tumor sites. Of these parameters, it was found surprisingly, that tumor staging of SOM was lower than that of the non-SOM and also that SOM was related to the uptake of TB at the prior cancer site and to the presence of OPLs at the previously treated cancer site (unpublished data).

As mentioned before, the literature has indicated that tumors of advanced stages are more likely to recur. Our results showing that tumor stages of SOM are significantly lower in stage were contrary to the literature. For this reason, the treatment of different stages of tumor were compared (Table VI-3). The results showed that the majority (93%) of

stage 0 (*CIS*) tumors were treated surgically, the majority of stage 3 and 4 (83%) tumors were treated with radiation (42% with surgery, and 42% alone). Of those tumors treated surgically, *CIS* were also more likely to be treated with laser surgery compared to other surgically treated invasive SC which were more likely to have wide excisions. These results would suggest that the treatment of *CIS* and perhaps early invasive SCC (stage 1 and 2) may not have been aggressive enough perhaps leaving behind tumor cells or severe dysplastic cells to outgrow into the SOM after the treatment.

VII.2. LOH and SOM

The major emphasis of this thesis was to study the molecular characteristics of target samples from the prior (index) cancer site in a longitudinal study setting, and to confirm and validate the results from retrospective study in our laboratory. The validation of an additional tool to identify OPLs at high-risk for the development of SOM is particularly important, since histology, the gold standard, has a reduced predictive value in comparison to primary cancers since treatment of the initial cancer with surgery and radiation frequently produces reactive tissue changes that could resemble dysplasia.

One of the major molecular tools is the use of microsatellite analysis of LOH since it uses only a small amount of DNA, an idea situation for the study of OPLs, which are usually small. Recent studies from a number of laboratories have shown that high-risk OPLs are characterized by elevated LOH frequencies, most often on multiple arms (Califano *et*

al., 1996; Mao *et al.,* 1996; Rosin *et al.,* 2000, 2002; Partridge *et al.*, 2001). A landmark study from this lab has shown that LOH at 3p and/or 9p is an early step for oral carcinogenesis and for cancer progression, with additional losses at other arms markedly increasing risk for primary OPLs (3.8-fold increase in relative cancer risk). A retrospective study from this lab also showed that lesions with LOH at 3p &/or 9p had a 26.3-fold increase in the risk of developing SOM.

This thesis describes early results from an ongoing prospective study of 86 patients with a history of oral cancer, in which LOH was evaluated for its ability to facilitate the identification of lesions with high risk of progression into SOM. The preliminary results suggest that LOH profile of lesions could be used as a powerful tool to identify the high-risk lesions.

The most significant finding of this thesis is that SOM lesions contained increased LOH for all categories studied, with many of these increases statistically significant. These patterns include any loss, multiple losses (> 1 arm and > 2 arms lost), LOH at individual chromosome arm 3p, 4q, 9p and 17p, LOH for 3p &/or 9p plus any of the other chromosomes, and simply LOH from 3p &/or 9p (without consideration of any other arm). The latter pattern represents the highest molecular risk pattern studied in this thesis. Survival analysis shows a 21.4-fold increase ($P < 0.0001$) in risk of developing SOM for patients with 3p and/or 9p loss and a 21.8-fold increase ($P < 0.0001$) in SOM risk associated with the LOH pattern of 3p and/or 9p loss plus allelic loss at other arms. Interestingly, the predictive value does not show improvement with incorporation of data on the loss at other arms, which is consistent with the result of previous prospective study

performed in our lab. Taken together, all these results lead to a conclusion that LOH on 3p14.2 and/or 9p21 can be utilized to screen patients at high risk of developing SOM after the treatment of the previous carcinoma.

VII.3. The value of using exfoliated brushing cell samples

Current molecular analysis mostly relies on biopsy of visible lesions. If a biopsy is not taken, no matter how powerful the molecular markers are in the prediction of SOM, we would not be able to predict the SOM.

Taking biopsies relies on clinician's ability to recognize that a lesion may be at risk for cancer. The recognition of lesions from previous cancer sites could be very difficult since treatment-induced reactive lesions could easily be confused with OPLs, even by experienced specialists. In addition, as previously stated, clinicians are also reluctant to biopsy a fragile treated sites repeatedly because of compromised healing. Furthermore, high-risk lesions may not be clinically visible. This longitudinal study indeed showed that biopsies of the prior cancer sites were only done very infrequently. Noninvasive methods that could be used to collect samples for molecular analysis are highly desired. Studies from this laboratory had shown that exfoliative cells taken by scraping/brushing a lesion showed similar LOH pattern as concordant biopsy samples, suggesting that brushings could be used as a DNA source to monitor SOM.

In this thesis, the majority of target samples (57/89) were brushings. The study showed that brushings could serve as a DNA source for LOH. Brushings from SOM cases showed significantly higher frequencies of LOH at 3p &/or 9p (P = 0.0074). This is a very exciting finding since this would suggest that we could collect samples from known high-risk regions (e.g., prior cancer site) noninvasively, which would allow for more intensive follow-up by molecular analysis. As a result, by thoroughly examining the profile of genetic alternations in these cells, we could identify the presence of residual cancer cells and genetically altered preneoplastic cells in a high-risk field, even when there is no obvious clinical lesion or when the clinical lesion appears too innocuous to warrant a biopsy.

It should be noted that technology advancement could further enhance the molecular analysis using brushing, which contains only minute amounts of DNA. Currently most of LOH analysis of scrapes used DNA amplified with PEP. The PEP technique is not ideal as amplified DNA may not show totally identical LOH results to straight DNA.

In summary, exfoliative cytology is becoming increasingly important in the early diagnosis of oral cancer, as a procedure for obtaining cell samples that can be analyzed by sophisticated diagnostic techniques such as DNA cytometry and microsatellite analysis. Exfoliative cytology is simple and rapid, non-aggressive and relatively painless: it is thus well-accepted by patients and suitable for routine application in population screening programs, for early analysis of suspect lesions or areas, and for post-treatment monitoring of high-risk lesions or areas.

VII.4. Tumor recurrences and SPTs

The differentiation of tumor recurrence from SPT from a prior tumor site could be difficult. Currently if a tumor developed from the previous tumor site (within the site or within 2 cm from the previous tumor site), it is called recurrence if the tumor occurs within 3 years of the prior tumor (some studies use 5 years), with SOMs developing beyond 3 years called SPTs. In real life, this is not that simplistic since in theory a SPT could develop within 3 years of prior tumor treatment. For this reason, our research team has intentionally chosen not to label a particular tumor as recurrence or SPT by the recurrence time.

Differentiation of recurrence from SPT, however, does have clinical implication. The former suggest that treatment has been inadequate, and the latter that the SOM is a new cancer developing independently. Recurrence and SPT may also differ in biological behavior.

For the above reasons, this thesis also tried to determine whether the target sample is a genetic outgrowth of the index tumor. There are three potential scenarios for genetic changes at the prior cancer site: (1) the target lesion derived from the genetic clones left by the index tumor (recurrence); (2) the target lesion derived from the genetic clones of the dysplastic lesions adjacent to the index tumor (recurrence, or field tumor as suggested

by some); (3) the target lesion was a new lesion that was not related to the index lesion (SPT).

In scenario one, the genetic changes of the target sample should be identical (see TableVI-9 for category 'Same') or near identical to the index tumor (see category 'Maybe the same'). The latter could result from the treatment effect. In addition, the progenitor population may not be homogeneous and therefore the outgrowth of a more dominant subclone would lead to an allelotype that had most but not all alterations of the index tumors, yet still represent the recurrence of the initial population.

In Scenario two, the target sample and the index lesion may share the early genetic changes but not the late changes since the index tumor may have additional changes compared to the adjacent dysplasia. For this reason, TableVI-9 has compared the SOM and non-SOM using three steps: 1st, earliest losses 3p and 9p; then add the next group of losses (3p, 9p, 4q and 17p), and finally include the late changes of 8p, 11q and 13q.

In scenario three, the target sample and the index lesion should have a good likelihood to be different in their losses (see Section VI.4. for criteria).

The study results showed that target samples from SOM were more likely to derived from left over genetic clones (recurrences, suggesting inadequate removal): target samples from SOM showed a consistently higher percentage of lesions demonstrating the same or near same genetic changes for all three comparisons, i.e., comparison of early changes

(3p and 9p), comparison of early and intermediate changes (3p, 9p, 4q, and 17p) and comparison of all (all $P < 0.05$). The latter would indicate that the genetic profile of the target sample was identical to the index tumor.

All of these findings strongly support the suggestion that the outgrowth of residual cancer cells or premalignant cells at the previous cancer site is largely responsible for new invasive carcinoma developing after the treatment of primary carcinoma. Meanwhile, the data provides additional molecular explanation for the clinical observation that there is an increased likelihood of tumor recurrence in patients with a history of oral cancer. Therefore, it is necessary for care providers to be more diligent about monitoring and screening high-risk lesions or areas in high-risk patients (those who have a history of oral cancer), even though there are no visual lesions observed clinically. In the future, the presence of residual cancer cells or genetically altered premalignant cells at mucosa defined by molecular analysis to be high-risk may be an indication for post-treatment, including chemotherapy or radiotherapy, to treat altered clonal lesions or areas that escape the histopathological detection and are beyond the initial scope of surgical excision.

VII.5. Limitations of the study and future plan

Microsatellite analysis in this study has shown a markedly high LOH frequency in tissue samples taken from the previous cancer site to be predicative of development of SOM

after the treatment of primary carcinoma. The clinical implication of this study is that it provides further support for the establishment of new follow-up protocols that incorporate molecular analysis into the routine examination for SOM risk assessment, especially for those people with a history of oral cancer.

However, before the results can be applied clinically to guide patient management, the study data must be further tested and confirmed in a larger sample size and with other populations in other laboratories. Due to the time restraint for a MSc thesis, to date, this longitudinal project could only study 89 patients, which consequently makes the power of study as 0.65 (see section V.1.6). Therefore, larger sample numbers are needed to minimize the errors of statistic analysis and increase the study power.

For future plans, a large-scale prospective study will be needed to confirm the study results. If prospective studies with large number of patients confirm my study findings, the future management of oral cancer patients with residual low-grade dysplastic cells or cancer cells may be dramatically changed, which could have a significantly positive impact on early diagnosis of SOM after the treatment of the primary cancer to improve the dismal 5-year survival rate.

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APPENDIX RAW DATA FILE

Study ID	End point	DOB (d/m/y)	Gender	Ever Smoke	Biopsy or Scrape	Presence of lesion	Homo-lesion	BX date of target tumor (d/m/y)	Date of tumor tx (d/m/y)	Target sample date (d/m/y)	Date of recur (d/m/y)	Date of last scrape (d/m/y)	Tumor stage	Histo-grade	TB staining
1003	no rec	17-06-42	F	yes	B	no	yes	09-08-94	17-02-95	28-10-98	NA	12-07-04	1	1	pos
1011	no rec	19-01-36	F	yes	S	no	no	02-09-98	02-09-98	29-03-00	NA	11-11-04	1	1	neg
1018	no rec	12-06-37	F	yes	S	yes	yes	10-10-96	05-08-97	11-12-97	NA	21-04-98	1	1	NA
1019	no rec	01-12-60	M	yes	B	yes	NA	23-03-94	03-06-94	20-07-95	NA	11-11-04	2	1	NA
1020	no rec	03-05-43	M	yes	S	yes	yes	21-10-97	21-05-99	20-01-00	NA	11-11-04	1	1	equiv
1021	no rec	02-10-44	M	yes	S	no	no	07-04-98	23-06-99	21-02-00	NA	11-11-04	1	2	neg
1028	no rec	26-09-24	F	no	S	no	no	25-11-98	30-05-01	27-03-02	NA	15-09-04	CIS	CIS	neg
1031	no rec	27-08-11	F	yes	S	yes	no	10-03-95	20-04-95	16-09-96	NA	11-11-04	1	1	NA
1039	no rec	13-10-13	F	no	S	no	no	26-07-95	26-07-95	31-05-96	NA	10-03-04	NA	1	neg
1042	no rec	08-08-55	F	no	B	yes	no	25-06-93	01-02-98	07-05-98	NA	11-11-04	1	1	pos
1050	no rec	07-12-42	M	yes	S	no	no	19-12-02	19-12-02	02-07-03	NA	13-10-04	1	2	neg
1054	no rec	15-06-40	M	yes	S	yes	yes	15-01-99	15-01-99	04-08-99	NA	15-09-04	1	1	equiv
1068	no rec	28-04-48	F	no	S	yes	NA	28-09-98	28-09-98	09-12-98	NA	11-11-04	1	2	NA
1074	no rec	04-02-41	M	yes	S	yes	yes	26-08-98	21-09-98	09-02-99	NA	29-03-02	CIS	CIS	neg
1076	no rec	12-11-30	F	yes	S	no	no	25-09-01	05-11-01	09-08-02	NA	11-11-04	2	1	NA
1078	no rec	10-03-63	F	yes	S	yes	yes	27-07-94	27-07-94	17-06-99	NA	11-11-04	1	1	neg

Study ID	End point	DOB (d/m/y)	Gender	Ever Smoke	Biopsy or Scrape	Presence of lesion	Homo-lesion	BX date of target tumor (d/m/y)	Date of tumor tx (d/m/y)	Target sample date (d/m/y)	Date of recur (d/m/y)	Date of last scrape (d/m/y)	Tumor stage	Histo-grade	TB staining
1080	no rec	07-08-50	M	yes	S	no	no	02-12-97	01-12-97	13-02-98	NA	29-06-02	CIS	CIS	neg
1082	no rec	01-04-42	F	no	S	no	no	18-05-01	22-06-01	25-03-02	NA	21-06-04	1	1	NA
1094	no rec	12-02-80	M	no	S	no	no	09-03-98	26-05-98	18-12-98	NA	28-12-01	2	1	neg
1098	no rec	20-10-42	F	yes	S	no	no	02-06-99	12-07-99	22-02-01	NA	08-11-04	1	2	neg
1103	no rec	02-01-18	F	yes	S	yes	yes	14-01-99	17-02-99	27-01-00	NA	06-05-01	1	1	neg
1106	no rec	26-03-12	F	no	B	yes	yes	13-04-99	01-06-99	11-08-99	NA	11-11-04	1	1	NA
1109	no rec	17-02-37	M	yes	B	yes	NA	24-03-94	24-03-94	15-08-94	NA	22-06-04	CIS	CIS	NA
1111	no rec	22-10-49	M	no	S	no	no	12-12-96	10-01-97	29-10-01	NA	11-11-04	CIS	CIS	neg
1120	no rec	12-12-48	M	no	S	yes	yes	18-02-97	27-03-97	19-07-99	NA	11-11-04	1	2	NA
1134	no rec	25-04-27	M	yes	S	yes	yes	04-01-99	06-08-99	10-12-99	NA	24-03-02	CIS	CIS	neg
1146	no rec	24-05-23	M	yes	S	no	no	15-04-99	02-06-99	21-02-00	NA	25-10-01	2	1	neg
1158	no rec	15-10-66	F	no	S	no	no	10-10-96	24-02-97	30-10-97	NA	11-11-04	1	1	NA
1159	no rec	29-04-53	F	no	S	no	no	04-06-98	25-06-98	31-05-99	NA	11-11-04	1	1	NA
1175	no rec	16-10-34	M	yes	S	yes	yes	08-01-98	07-03-98	25-02-99	NA	11-11-04	1	1	NA
1177	no rec	31-12-19	F	yes	S	yes	yes	23-10-98	08-04-99	17-07-00	NA	08-09-04	1	1	equiv
1179	no rec	21-03-38	F	yes	S	no	no	09-02-00	15-06-00	22-01-01	NA	11-11-04	2	1	neg
1179	no rec	21-03-38	F	yes	S	no	no	19-02-93	19-02-93	12-09-00	NA	11-11-04	1	1	neg

Study ID	End point	DOB (d/m/y)	Gender	Ever Smoke	Biopsy or Scrape	Presence of lesion	Homo-lesion	BX date of target tumor (d/m/y)	Date of tumor tx (d/m/y)	Target sample date (d/m/y)	Date of recur (d/m/y)	Date of last scrape (d/m/y)	Tumor stage	Histo-grade	TB staining
1180	no rec	09-11-28	F	yes	B	yes	NA	10-03-99	10-03-99	12-09-00	NA	11-11-04	1	1	neg
1185	no rec	23-08-46	F	yes	S	yes	yes	29-04-02	30-05-02	11-12-02	NA	02-11-04	CIS	CIS	neg
1203	no rec	18-09-55	F	no	B	yes	NA	24-09-99	24-09-99	26-03-01	NA	11-11-04	CIS	CIS	equiv
1213	no rec	03-08-57	M	no	B	yes	NA	25-05-99	25-05-99	02-09-99	NA	15-09-04	2	1	NA
1215	no rec	16-10-44	M	no	S	yes	yes	17-04-97	13-07-97	24-07-00	NA	22-08-01	2	2	neg
1223	no rec	12-11-42	F	yes	S	no	no	06-01-00	06-01-00	24-07-00	NA	11-11-04	CIS	CIS	equiv
1226	no rec	14-11-25	M	yes	S	no	no	11-02-00	29-02-00	16-11-00	NA	01-01-02	CIS	CIS	neg
1231	no rec	28-07-53	F	1	B	yes	no	12-08-99	15-10-99	15-06-00	NA	11-11-04	1	1	equiv
1235	no rec	10-03-16	F	1	S	no	no	25-01-00	20-03-00	06-02-01	NA	17-07-02	2	1	neg
1236	no rec	22-09-36	M	1	S	no	no	15-11-00	15-11-00	01-05-02	NA	11-11-04	1	1	neg
1246	no rec	27-01-40	F	no	B	yes	yes	26-08-83	15-09-83	26-04-00	NA	11-11-04	CIS	CIS	neg
1252	no rec	12-12-59	M	1	S	no	no	09-02-00	29-03-00	16-11-00	NA	11-11-04	1	1	neg
1262	no rec	15-01-55	F	no	B	yes	NA	08-11-99	09-12-99	24-07-00	NA	11-11-04	CIS	CIS	neg
1269	no rec	26-10-48	F	1	B	yes	yes	28-03-03	27-05-03	31-07-03	NA	11-11-04	1	1	pos
1270	no rec	10-03-32	F	1	S	no	no	21-07-00	30-08-00	01-11-01	NA	11-11-04	2	2	equiv
1280	no rec	29-04-62	F	no	S	no	no	05-09-01	05-09-01	25-03-02	NA	01-05-03	CIS	CIS	neg
1281	no rec	21-04-23	F	no	S	yes	no	29-05-00	09-05-00	28-11-01	NA	11-11-04	NA	NA	pos

Study ID	End point	DOB (d/m/y)	Gender	Ever Smoke	Biopsy or Scrape	Presence of lesion	Homo-lesion	BX date of target tumor (d/m/y)	Date of tumor tx (d/m/y)	Target sample date (d/m/y)	Date of recur (d/m/y)	Date of last scrape (d/m/y)	Tumor stage	Histo-grade	TB staining
1303	no rec	23-11-31	F	1	B	yes	NA	24-01-01	24-01-01	17-10-01	NA	11-11-04	1	2	neg
1308	no rec	26-03-34	F	no	S	no	no	18-10-00	21-12-00	19-12-01	NA	11-11-04	1	1	neg
1311	no rec	15-05-50	M	1	S	no	no	20-09-00	10-10-00	23-04-01	NA	21-07-04	CIS	CIS	neg
1321	no rec	06-05-51	F	1	S	no	no	09-02-01	23-04-01	27-03-02	NA	11-11-04	CIS	CIS	neg
1326	no rec	01-05-32	M	NA	B	yes	NA	17-10-84	09-01-85	01-05-86	NA	07-02-91	2	1	NA
1334	no rec	15-11-53	M	1	S	yes	yes	28-09-00	06-12-00	13-08-01	NA	11-11-04	2	2	neg
1340	no rec	01-06-54	M	1	B	yes	no	12-10-95	29-11-95	12-11-02	NA	11-11-04	1	1	pos
1344	no rec	24-09-33	M	1	B	yes	NA	02-11-00	13-12-00	05-03-01	NA	10-02-04	2	1	neg
1356	no rec	03-01-40	M	1	S	no	no	16-05-01	16 05 01	11 01 02	NA	11 11 04	1	1	neg
1364	no rec	21-05-50	F	no	S	no	no	16-07-01	05-09-01	10-06-02	NA	11-11-04	1	1	neg
1367	no rec	18-07-22	F	no	S	no	no	16-08-01	16-08-01	24-07-02	NA	18-10-04	CIS	CIS	neg
1396	no rec	04-03-33	F	no	S	no	no	05-12-01	17-01-02	06-05-02	NA	11-11-04	1	1	neg
1410	no rec	11-03-46	F	1	S	no	no	26-10-01	08-01-02	13-02-02	NA	23-08-04	CIS	CIS	neg
1421	no rec	30-12-44	F	no	S	no	no	07-03-02	07-03-02	20-11-02	NA	11-11-04	CIS	CIS	neg
1004	rec	08-08-49	F	no	B	yes	NA	08-01-97	08-01-97	30-03-98	08-01-99	01-03-01	1	1	NA
1009	rec	03-12-30	M	1	B	yes	yes	23-02-99	13-07-99	18-11-99	19-09-01	01-12-03	CIS	CIS	equiv
1078	rec	10-03-63	F	1	B	yes	NA	11-12-81	28-05-88	25-07-88	30-06-94	11-11-04	1	1	equiv

Study ID	End point	DOB (d/m/y)	Gender	Ever Smoke	Biopsy or Scrape	Presence of lesion	Homo-lesion	BX date of target tumor (d/m/y)	Date of tumor tx (d/m/y)	Target sample date (d/m/y)	Date of recur (d/m/y)	Date of last scrape (d/m/y)	Tumor stage	Histo-grade	TB staining
1079	rec	09-03-27	M	no	B	yes	yes	17-04-98	17-04-98	16-09-98	10-10-03	09-10-03	1	1	pos
1099	rec	01-03-55	M	1	B	yes	NA	15-02-94	15-02-94	19-09-94	03-10-96	04-12-96	NA	1	NA
1107	rec	04-01-18	F	no	S	yes	no	09-03-98	27-05-98	03-02-99	25-03-99	11-11-04	CIS	CIS	equiv
1110	rec	02-01-45	M	1	B	yes	no	25-11-96	17-12-96	29-07-99	13-01-00	11-11-04	1	1	NA
1112	rec	06-02-18	F	1	B	yes	no	06-04-95	21-03-96	23-05-97	01-10-98	10-06-03	1	1	pos
1118	rec	19-11-37	F	no	B	yes	yes	25-04-89	18-05-89	27-11-95	07-10-97	07-10-97	1	2	NA
1132	rec	24-12-42	M	1	B	yes	yes	28-02-95	28-02-95	29-07-96	22-04-98	20-12-99	CIS	CIS	pos
1142	rec	02-05-57	M	1	B	yes	NA	14-09-88	09-04-91	09-04-91	26-06-92	13-09-00	1	1	NA
~~1145~~	~~rec~~	~~25-01-34~~	~~M~~	~~1~~	~~B~~	~~yes~~	~~yes~~	~~08-04-99~~	~~08-04-99~~	~~17-11-99~~	~~26-06-00~~	~~23-01-04~~	~~CIS~~	~~CIS~~	~~pos~~
1145	rec	25-01-34	M	1	B	yes	yes	19-01-96	15-02-96	23-02-98	08-04-99	23-01-04	CIS	CIS	equiv
1148	rec	13-09-53	F	1	S	yes	yes	08-03-99	01-05-99	07-06-00	04-04-01	03-11-04	CIS	CIS	equiv
1156	rec	21-03-50	F	no	B	yes	NA	15-02-90	15-02-90	29-01-96	22-07-98	22-07-98	1	1	NA
1192	rec	02-06-23	M	1	B	yes	no	28-07-99	28-07-99	29-10-99	16-03-00	06-10-04	1	1	equiv
1195	rec	13-03-20	M	no	S	yes	yes	04-10-99	26-11-99	20-06-00	20-03-03	20-10-04	1	1	neg
1197	rec	16-03-41	M	1	S	no	no	22-06-00	22-06-00	04-12-00	18-01-01	11-11-04	CIS	CIS	neg
1212	rec	28-07-45	M	1	S	yes	NA	24-03-99	10-09-99	28-09-00	27-11-01	26-07-04	CIS	CIS	neg
1280	rec	29-04-62	F	no	S	no	no	24-08-00	24-08-00	23-04-01	05-09-01	21-03-04	1	1	neg

1300	rec	29-05-17	M	1	B	yes	no	09-09-94	01-09-94	11-12-00	22-01-04	22-01-04	1	1	equiv
1307	rec	03-06-58	M	1	S	yes	yes	14-12-00	25-01-01	16-05-01	22-08-01	11-11-04	CIS	CIS	neg
1325	rec	03-12-27	F	no	B	yes	NA	18-12-00	27-07-01	19-02-02	08-05-02	11-11-04	CIS	CIS	NA
1349	rec	23-02-46	F	1	S	yes	yes	03-04-01	03-04-01	27-05-02	22-09-03	11-11-04	CIS	CIS	neg
1388	rec	01-10-15	M	1	S	yes	no	29-10-01	06-12-01	24-04-02	19-08-02	14-06-04	1	1	pos

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1003	no rec	S	no	R	R	R	R	R	R	R
1011	no rec	S	no	R	L	R	R	R	R	R
1018	no rec	R	no	R	R	NI	R	L	R	R
1019	no rec	R	no	R	R	R	L	R	L	L
1020	no rec	R	no	R	L	R	L	R	L	R
1021	no rec	S & R	no	R	L	R	L	R	R	R
1028	no rec	S	yes	L	NI	R	R	R	R	R
1031	no rec	S	no	R	R	R	R	R	R	R
1039	no rec	S	no	R	NI	R	L	R	R	L
1042	no rec	S	yes	R	R	R	R	R	R	R
1050	no rec	S	no	R	R	R	L	L	L	L

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1054	no rec	S	no	R	R	R	R	R	R	R
1068	no rec	S	no	R	R	R	R	R	R	R
1074	no rec	S	yes	R	ND	ND	R	ND	ND	R
1076	no rec	R	no	R	R	R	R	R	R	R
1078	no rec	S	no	R	R	R	R	R	R	R
1080	no rec	S	yes	R	ND	ND	R	ND	ND	R
1082	no rec	S & R	no	R	R	R	R	R	R	R
1094	no rec	S & R	no	R	R	R	R	R	R	R
1098	no rec	S	no	R	R	R	L	R	ND	R
1103	no rec	S	no	L	R	R	L	L	R	R
1106	no rec	R	no	L	R	R	L	R	R	R
1109	no rec	S	no	R	R	L	R	R	R	R
1111	no rec	S	no	R	R	R	R	R	R	R
1120	no rec	S	no	L	L	R	R	R	R	R
1134	no rec	R	no	R	R	R	L	NI	R	R
1146	no rec	R	no	R	ND	ND	R	ND	ND	R
1158	no rec	S & R	no	R	R	R	R	L	R	R

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1159	no rec	S	no	R	R	R	R	R	R	R
1175	no rec	S & R	yes	L	R	R	R	L	R	R
1177	no rec	R	no	R	R	R	R	R	R	R
1179	no rec	S & R	no	R	R	R	R	R	R	R
1179	no rec	S & R	no	L	R	R	R	R	R	R
1180	no rec	S	no	R	R	R	R	R	R	R
1185	no rec	S	yes	L	R	R	L	L	R	R
1203	no rec	S	no	R	R	R	R	R	R	L
1213	no rec	S	no	R	R	R	R	R	R	R
1215	no rec	S & R	no	R	R	R	R	R	ND	R
1223	no rec	S	no	R	R	R	R	R	R	L
1226	no rec	S	no	L	L	R	R	R	R	R
1231	no rec	R	no	R	R	R	R	R	R	R
1235	no rec	R	no	R	R	R	R	R	R	R
1236	no rec	S	no	R	R	R	R	R	R	R
1246	no rec	S	no	R	R	R	R	R	R	R
1252	no rec	S	no	R	R	R	R	R	R	R

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1262	no rec	S	yes	R	R	R	R	R	R	R
1269	no rec	S	yes	R	R	R	L	R	R	R
1270	no rec	S	no	R	R	R	R	R	R	R
1280	no rec	S	no	R	R	R	R	R	R	R
1281	no rec	NA	NA	R	R	R	R	R	R	R
1303	no rec	S	no	R	NI	R	R	R	R	R
1308	no rec	S	no	R	ND	NI	R	ND	R	R
1311	no rec	S	no	R	ND	L	R	ND	ND	R
1321	no rec	S	no	R	L	L	L	R	R	L
1326	no rec	R	no	L	R	R	L	NI	R	L
1334	no rec	S & R	no	R	L	R	R	R	R	R
1340	no rec	S	no	R	R	R	R	R	R	R
1344	no rec	S & R	no	R	R	R	R	R	R	R
1356	no rec	S	no	L	R	R	R	R	R	R
1364	no rec	S	no	R	R	R	L	R	R	R
1367	no rec	S	no	R	NI	R	R	R	L	R
1396	no rec	S	no	R	R	R	R	R	R	R

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1410	no rec	S	yes	R	R	R	R	R	R	R
1421	no rec	S	no	R	R	R	R	R	R	R
1004	rec	S	no	L	L	R	L	R	R	L
1009	rec	S	yes	R	R	L	L	R	R	L
1078	rec	S & R	no	R	L	R	L	R	L	R
1079	rec	S	no	R	NI	R	L	R	NI	R
1099	rec	S	yes	R	NI	L	L	R	R	L
1107	rec	R	no	L	L	R	L	L	L	R
1110	rec	S	yes	L	R	R	R	L	L	R
1112	rec	S	no	L	L	L	L	L	R	L
1118	rec	S	no	R	L	L	L	R	R	R
1132	rec	S	no	R	R	R	L	R	R	L
1142	rec	R	no	R	L	R	L	R	R	L
1145	rec	S	no	L	SH	R	L	R	R	L
1145	rec	S	no	L	SH	R	L	R	R	L
1148	rec	S	yes	R	R	R	R	R	R	R
1156	rec	R	no	L	R	R	L	R	R	R

Study ID	End point	TX of target tumor	Tumor treated with laser	3p LOH	4q LOH	8p LOH	9p LOH	11q LOH	13q LOH	17p LOH
1192	rec	S	no	L	SH	R	L	R	R	R
1195	rec	R	no	R	ND	ND	L	ND	ND	R
1197	rec	S	yes	L	R	R	R	R	NI	R
1212	rec	S	yes	L	R	R	R	L	R	R
1280	rec	S	no	R	R	R	R	R	R	R
1300	rec	S	no	R	L	R	L	R	R	L
1307	rec	S	no	R	R	R	R	L	R	R
1325	rec	S	no	L	R	R	L	R	R	L
1349	rec	S	no	L	R	R	L	R	NI	L
1388	rec	S	no	R	R	R	R	R	R	R