MICROSATELLITE STUDIES OF ORAL CANCER AND PREMALIGNANT LESIONS

by

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We accept this thesis as conforming to the required standard

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Date **Sept 14, 2001**
ABSTRACT

Oral squamous cell carcinoma (SCC) is believed to progress through sequential stages of premalignancies to invasive cancer. Once SCC is formed, the prognosis is poor. The 5-year survival rate of less than 50% is one of the worst among major human cancers. One of the essential keys to improving this gloomy prognosis lies in early diagnosis and proper management of premalignant lesions.

A central dogma of carcinogenesis is that alteration to critical control genes underlies malignant transformation. A major focus of this thesis was to study early molecular pathways of oral cancer development and their impact on treatment. Loss of heterozygosiy (LOH) has been shown to be a powerful molecular approach to detect genetic alteration of tumor suppressor genes in many organs and systems, including oral cancer and premalignant lesions. It is also the major molecular technique used in this thesis.

Genetic changes in oral premalignant lesions and SCC studied (n=226) were investigated by microsatellite analysis for LOH at 19 loci on chromosome arms (3p, 9p, 17p, 4q, 8p, 11q, and 13q).

This thesis has provided data to establish molecular progression models for both SCC and VC, which provide information on molecular cancer risk of premalignancies, and has yielded the first evidence that premalignancies with different molecular risks could be managed differently. It has also shown a possible role of HPV in the development of posttransplant oral SCC. These results not only provide insight into the mechanism of early cancer development but also suggest the use of these molecular assays as tools for targeting high-risk premalignancies. Hence, the studies have important clinical implications. The importance of the data is illustrated by the fact
that a recent editorial on latest advances on genetic studies of oral premalignancies from the *New England Journal of Medicine* (Lippman and Hong, 2001) has cited 8 significant studies and one of these is from this thesis, and another from this lab with data from this thesis.
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LIST OF ABBREVIATIONS

CIN    cervical intraepithelial neoplasia
CIS    carcinoma in situ
DNA    deoxyribonucleic acid
FHIT   fragile histidine triad
H & E  hematoxylin and eosin
HNSCC  head and neck squamous cell carcinoma
HPV    human papillomavirus
GAPDH  glyceraldehyde phosphate dehydrogenase
GVHD   graft versus host disease
LOH    loss of heterozygosity
NTP    non-transplant patient
OPL    oral premalignant lesion
ORF    open reading frame
PCR    polymerase chain reaction
OR     odds ratio
SCC    squamous cell carcinoma
TBI    total body irradiation
TP     transplant patient
TSG    tumor suppressor gene
VC     verrucous carcinoma
VH     verrucous hyperplasia
WHO    World Health Organization
FOREWORD ON THESIS FORMAT

This thesis consists of six chapters. The first two chapters include a general introduction (Chapter I), and an overview of basic aspects of study design and methodology (Chapter II). These chapters are followed by three more chapters, each including an article in its publication format or in its submission format (Chapters III-V). The thesis ends with a chapter that contains more studies and general discussion (Chapter VI).

It should be noted that my study is focused on clinical problems. Clinical research is extremely labor intensive, requiring not only extensive time in the laboratory, but also time in hospitals, with the difficult tasks of searching for samples, database management, and chart review. As these processes are lengthy, only part of my work is completed and is presented here in these three papers. Some of my contributions to the research projects of the research team have already been published with me as a coauthor and are not presented here as part of my thesis (Rosin et al., 2000; Zhang et al., 2000, 2001b). I will also be a coauthor on several studies that are still ongoing (Zhang et al., 2001c, Rosin et al., 2001; Epstein et al., submitted; Zhang et al., in preparation).

More details on the composition of each of the six chapters in this thesis follow.

Chapter I gives a general review of the literature to provide background information on the clinical problems that are the focus of this thesis as well as the rationale for choosing the study topics for the 3 papers. It includes a detailed review of contemporary molecular knowledge on carcinogenesis, especially on loss of heterozygosity (LOH) in oral premalignant lesions and oral cancers, the primary assay used in this thesis.
Chapter II describes study approaches and molecular methods used in the three papers. This includes details on the microsatellite assay for LOH, a PCR-based assay used to detect human papillomavirus (HPV) infection and the $p53$ mutation assay.

Chapter III presents an article that has been published by *Laboratory Investigation* (2001, 81: 629-34), which ranks the second highest in impact factors among pathology journals. This paper is the first to investigate the genetic pathway underlying development and progression of oral verrucous lesions. LOH patterns in verrucous lesions (verrucous hyperplasia and carcinoma) were compared with those of dysplasia and SCC to determine the similarities and differences between these two groups of closely related lesions. LOH patterns were also compared to those of reactive hyperplasias to determine whether microsatellite analysis could facilitate the differential diagnosis of verrucous lesions from reactive hyperplasias. Differentiation of these two lesions presents a diagnostic challenge for pathologists. Improper diagnosis has a drastic impact on patient management and prognosis. It should be noted that the extensive LOH analysis (19 primers on 7 chromosome arms) of dysplasia, squamous cell carcinoma (SCC), and reactive hyperplasia in this study, together with prior data from other members of the research team, has generated the first oral genetic carcinogenesis pathway that incorporates different degrees of dysplasia (see CHAPTER VI. MORE STUDIES AND GENERAL DISCUSSION) and has been used to establish three molecular risk patterns that are currently being tested in an ongoing longitudinal study at the British Columbia Cancer Agency (Rosin et al., 2001).

Chapter IV includes an article that has been accepted for publication in *Oral Oncology* (2001, 37: 505-512). It represents the first study to investigate the impact of treatment on oral premalignant lesions with different molecular risks. In this controversial but provocative study, data are presented that supports the use of the microsatellite assay not only in treatment
decisions, but also in the assessment of treatment efficacy, i.e. whether a lesion has been completely removed.

Chapter V contains an article that has been submitted to *Journal of Oral Medicine and Pathology*. This article examines the molecular mechanisms underlying the increased oral SCCS observed in patients with a previous history of transplantation. It compares molecular changes in these lesions to those in oral cancers from non-transplant patients. For post-transplant patients, 5 oral SCCS were assessed for LOH patterns, HPV infection and *p53* mutation. For patients with no history of transplantation, 34 oral SCCS were assessed for HPV infection; data on LOH for the 34 cases were taken from paper 1, and data on *p53* mutation were taken from the literature. The results showed a marked increase in HPV infection in post-transplant SCCS, suggesting a different etiology for such cancers.

Chapter VI presents a general discussion of topics and findings developed in this thesis. Two genetic progression pathways are presented, one for the “dysplasia to SCC” pathway and the other for the “verrucous hyperplasia to verrucous carcinoma” pathway. The overall relevance of the molecular information generated in this thesis to prevention of oral premalignant and malignant lesions is discussed. In addition, ongoing studies and future studies that need to be done are described.
ACKNOWLEDGEMENTS

I would like to express gratitude to Dr. Lewei Zhang and Dr. Miriam Rosin for giving me the opportunity to become a graduate student at the University of British Columbia and for allowing me to follow my interests. Their trust, encouragement and instruction has allowed me to complete this challenging work in a timely fashion. It is really a great honor and pleasure to work with them. I also appreciate the participation of Dr. Jukka Uitto and Dr. Gerardo Maupome as members of my supervisory committee, and I value the opinions and time taken by all members of my examining committee.

I would also like to take this opportunity to thank my mom and my children, Alice and Alex, for their continuous support and understanding.
DEDICATION

To Alice and Linda

Who love me and whom I love forever
CHAPTER I.

INTRODUCTION AND LITERATURE REVIEW
I.1. **Rationale for studying oral premalignant and malignant lesions, particularly premalignant lesions**

Oral squamous cell carcinoma (SCC) is believed to progress through sequential stages of oral premalignant lesions: hyperplasia to epithelial dysplasia of varying degree, to carcinoma *in situ* (CIS), and finally invasive cancer. It is the sixth most common cancer in the world accounting for about 3% of all new cancers in the Western world (Harras *et al.*, 1996; Greenlee *et al.*, 2001) and up to 40% of all cancers in other parts of the world, such as India (Saranath *et al.*, 1993).

Once invasive cancer is formed, the 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). Despite refinement of surgical techniques and adjuvant therapies, the survival rate has not improved in the last several decades. High local recurrence and formation of second primary malignancies are the major causes of this high mortality rate (Cooper *et al.*, 1989; Day and Blot, 1992; Lippman and Hong, 1989; Shikhani *et al.*, 1986, Table 1, Table 2). Survivors frequently have to endure serious cosmetic and/or functional compromise. The key to improving the dismal mortality and morbidity rates of oral SCC is to prevent the formation of these invasive carcinomas.
Table 1. Recurrence rate of oral cancer and HNSCC

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample size</th>
<th>% of recurring</th>
<th>F/U duration (month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Dersten et al., 1995</td>
<td>155</td>
<td>37%</td>
<td>-</td>
</tr>
<tr>
<td>Charuruks et al., 1996</td>
<td>46</td>
<td>47%</td>
<td>2-55</td>
</tr>
<tr>
<td>Shin et al., 1996</td>
<td>66</td>
<td>50%</td>
<td>-</td>
</tr>
<tr>
<td>Ball et al., 1997</td>
<td>24</td>
<td>42%</td>
<td>24</td>
</tr>
<tr>
<td>Dhooge et al., 1998</td>
<td>127</td>
<td>31%</td>
<td>30 (1-77)</td>
</tr>
<tr>
<td>Sardi et al., 2000</td>
<td>25</td>
<td>32%</td>
<td>22 (15-31)</td>
</tr>
</tbody>
</table>

F/U: follow-up

Table 2. Incidence of secondary primary malignancy of oral cancer and HNSCC

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample size</th>
<th>% second primary</th>
<th>F/U duration(month)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shikhani et al., 1986</td>
<td>1961</td>
<td>9.7%</td>
<td>-</td>
</tr>
<tr>
<td>Charuruks et al., 1996</td>
<td>46</td>
<td>22%</td>
<td>6-76</td>
</tr>
<tr>
<td>Shin et al., 1996</td>
<td>66</td>
<td>33%</td>
<td></td>
</tr>
<tr>
<td>Dhooge et al., 1998</td>
<td>127</td>
<td>13.5%</td>
<td>30 (1-77)</td>
</tr>
</tbody>
</table>

F/U: follow-up

During the last decade, molecular techniques of cancer studies have developed rapidly. The availability of new technologies has led to an interest in developing new approaches that can be used to identify high-risk lesions so that intervention regimes can be targeted to such lesions.
more effectively and prognosis improved. As a result, there has been a recent emphasis on the study of early phases of carcinogenesis (premalignant lesions) and an increased awareness of the importance of screening and management of the carcinogenesis process at early stages. The rationale underlying such studies is that the early identification and appropriate management of high-risk lesions might decisively help prevent cancer before it has time to develop (Silverman et al., 1996).

SCC also occurs in many other organs and is the most common human cancer. An oral carcinogenesis model, based on the histological progression of premalignant lesions to SCC, is an excellent model for studying squamous cell carcinogenesis throughout the body. Other factors that make it an attractive model for studying SCC include the ease of accessibility of the tissue for periodic study and the availability of archives of tissue samples in premalignant stages for molecular analysis. The study of oral cancer has played a premier role in the study of SCC and has become a paradigm for similar studies of epithelial cancers in other sites (Hong et al., 2000).

1.1.1. Anatomy and histology of 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.
1.1.2. Oral premalignant lesions and histological progression models for oral premalignant lesions

A premalignant or precancerous lesion has been defined by the World Health Organization as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart (Pindborg et al., WHO, 1997). It is generally believed that a premalignant lesion often precedes oral cancer. Clinically, oral premalignant lesions most frequently present as leukoplakia and erythroplakia (Axell et al., 1996).

1.1.2.1. Leukoplakia

Leukoplakia means “white patch” and 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 is not removed by rubbing. 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 appearance of leukoplakia affects the malignant risk of lesions. According to their clinical appearance, leukoplakias may be classified as either homogeneous or non-homogeneous. Homogeneous leukoplakia 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).

The site of leukoplakia also 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).

### 1.1.2.2. Erythroplakia

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. Erythroplakias are generally composed of either lesions that are already malignant or high-risk precancerous lesions (Bouguot and Ephros, 1995; Mashberg, 1977; Waldron and Shafer, 1975).
I.1.2.3. Cancer risk of oral premalignant lesions

Most oral premalignant lesions present clinically as leukoplakias (Pindborg et al., WHO, 1997) and the majority of these will not progress into cancer. The reported cancer risk for oral premalignant lesions varies widely, from as low as 0.13% to as high as 50% (Lumerman et al., 1995; Papadimitrakopoulou and Hong, 1997; Silverman et al., 1984). This huge variation in progression risk is primarily due to differences between studies in both follow-up times and patient characteristics. For example, the inclusion of a higher number of frictional keratosis, which will not progress, would decrease the rate of malignant progression in some studies (Waldron and Shafer, 1975). Leukoplakia and erythroplakia are two clinical lesions widely considered to be premalignant. However, using clinical features to classify lesions is difficult because they vary in appearance and are likely to be interpreted subjectively by the clinician. A histopathologic diagnosis is generally more indicative of premalignant change than clinically apparent alterations.

Several factors are known to influence the risk of oral premalignant lesions. The current gold standard is histology (presence or degree of dysplasia). However, most studies on the malignant transformation risk of oral premalignant lesions have been done on leukoplakias; that is, the cancer risk of oral premalignant lesions was judged by clinical information frequently without knowledge of dysplasia. Other factors which affect malignant risk include clinical appearance and site of the lesions (see above), as well as other less confirmed factors: the gender of the patient (females have increased risk), history of tobacco habit (leukoplakia in those who never smoked have increased risk), size of the lesions (the larger the lesion, the higher the risk), duration of the lesions (the longer the duration, the higher the risk) and candidiasis (those with Candida infection have increased risk) (van der Waal et al., 1997).
1.1.2.4. Histopathology of premalignant lesions and histological progression model of oral squamous cell carcinoma

As histology is the conventional gold criteria for judging cancer risk, a suspected oral premalignant lesion will frequently be biopsied and the risk of malignancy judged by histological evaluation of the biopsy for the presence and degree of histological changes called “dysplasia”. When a biopsy is taken, a leukoplakia often shows hyperkeratosis and/or epithelial hyperplasia (acanthosis) with or without epithelial dysplasia microscopically. The World Health Organization (Pindborg et al., 1997) has established the following characteristics as dysplasia: 1) loss of polarity of the basal cells, 2) the presence of more than one layer having a basaloid appearance, 3) increased nuclear-cytoplasmic ratio, 4) drop-shaped rete-ridges, 5) irregular epithelial stratification, 6) increased numbers of mitotic figures, 7) mitotic figures that are abnormal in form, 8) the presence of mitotic figures in the superficial half of the epithelium, 9) cellular and nuclear pleomorphism, 10) nuclear hyperchromatism, 11) enlarged nucleoli, 12) loss of intercellular adherence, and 13) keratinization of single cells or cell groups in the prickle cell layer.

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 up to two-thirds of the thickness of the epithelium. Severe dysplasia is a lesion in which the dysplastic cells have filled more than two-thirds but less than the entire thickness of the epithelium. 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 the dissemination of these cells to other sites through the lymphoid and circulatory systems are events associated with development of invasive SCC.

All studies to date have shown that leukoplakia with dysplasia has a higher malignant risk than leukoplakia without dysplasia (Waldron and Shafer, 1975; Lumerman et al., 1995). A large clinical study by Silverman et al. (1984) found that more than 36% of lesions with microscopic epithelial dysplastic features eventually underwent malignant transformation within an average of 7.2 years after presentation of leukoplakia. In contrast, leukoplakia without dysplasia only demonstrated a malignancy rate of 16%. The impact on malignant risk of the existence and degree of dysplasia is further demonstrated by studies from the uterine cervix and other systems and organs including skin and respiratory system (Boone et al., 1992; Braithwaite and Rabbitts, 1999; Geboes, 2000; Pinto and Crum, 2000; Shekhar et al., 1998).

Using these criteria, a histological progression model has been established for oral cancer (Figure 1). In this model, oral cancers progress through hyperplasia and increasing degree of dysplasia, mild, moderate and severe, to carcinoma in situ (CIS), and finally break through the basement membrane and become SCCs. Usually severe dysplasia and CIS are grouped together as high-grade dysplasia. This is because both of them are late stage, preinvasive lesions and the distinction between them is often difficult and does not appear to be of practical value in the management of oral mucosa (Pindborg et al., WHO, 1997).
Figure 1. Histological progression model of oral premalignant and malignant lesions

Normal » VH * VC * SCC

Figure 2. Histological progression model of verrucous lesions

(adapted from Hansen et al., 1985)

Normal → VH → VC → SCC
1.1.2.5. **Histopathology and histopathological progression model of verrucous carcinoma**

While most SCCs are believed to develop through the various stages of the aforementioned histological progression model of hyperplasia to dysplasia to SCC (Figure 1), verrucous carcinoma (VC), a variant of SCC, is thought to progress through a somewhat different histopathological pathway. VC is a low-grade carcinoma first defined by Ackerman in 1948 (Ackerman, 1948). VC has been reported in various parts of the body but is most common in the oral cavity (Kraus and Perezmesa, 1966), where it accounts for 2 to 12 percent of all carcinomas in the oral cavity (Bonnie and Rankin, 1988; Bouquot, 1998; Fonts *et al.*, 1969; Goethals *et al.*, 1963; Jacobson and Shear, 1972; Jordan, 1995; McCoy and Waldron, 1981; Schrader and Laberke, 1988; Shafer *et al.*, 1993). While dysplasia is the premalignant lesion for SCC, verrucous hyperplasia (VH) has been recognized as the premalignant lesion for VC (Adkins and Monsour, 1976; Batsakis *et al.*, 1982; Fisker and Philipsen, 1984; Grinspan and Abulafia, 1979; Kamath *et al.*, 1989; Shear and Pindborg, 1980; Tsiklakis and Wood, 1988). A histopathological progression model for VC was proposed by Hansen *et al.* (1985) as shown in Figure 2. In this model, oral cancers progress through hyperplasia to a sharp variety of VH, to a blunt variety of VH, and finally grow endophytically and become VC (Shear and Pindborg, 1980).

As a distinct variant of SCC, VC has unique features clinically and histologically and a different prognosis compared to conventional SCC (Table 3). Clinically, it is characterized by a slowly growing large warty or verrucous lesion, commonly seen on the buccal mucosa and gingiva (instead of tongue and floor of mouth for SCC) (Jacobson and Shear, 1972), and occurs mainly in elderly men (one decade older than SCC) (Tornes *et al.*, 1985). Histologically, VC is
characterized by strikingly benign-looking verrucous epithelium, and dysplasia is either absent or, if present, not pronounced. The verrucous epithelium grows both exophytically and endophytically by pushing the large bulbous epithelium deep into the connective tissue or even into bone, causing bone destruction. In this sense, this lesion is invasive and destructive. However, there is no breakage of the basement membrane of the epithelium as seen in SCC, so VC is not invasive in a strict sense (Prioleau et al., 1980). Prognostically, VC has an excellent 5-year survival rate (75% as compared to 40-50% for SCC) and is believed not to metastasize, since it lacks the ability to break the basement membrane, a prerequisite for metastasis to occur. However, these lesions are also characterized by persistent local recurrence, and with repeated recurrence, sometimes can progress into conventional SCC. In such cases, long-term prognosis of these lesions may still be dismal.
Table 3. A comparison between VC and SCC, in terms of clinical behavior, histology and prognosis

<table>
<thead>
<tr>
<th></th>
<th>Verrucous Carcinoma</th>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Clinical behavior</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>2-12% of oral cancers</td>
<td>Over 90% of oral cancers</td>
</tr>
<tr>
<td>Age at onset</td>
<td>60-70</td>
<td>50-60</td>
</tr>
<tr>
<td>Most common sites</td>
<td>Buccal and gum</td>
<td>Tongue and FOM(^1)</td>
</tr>
<tr>
<td>Possible etiology</td>
<td>Smokeless tobacco usage, human papillomavirus infection</td>
<td>Tobacco and alcohol habits, human papillomavirus infection?</td>
</tr>
<tr>
<td>Clinical appearance</td>
<td>Usually exophytic verrucous configuration</td>
<td>Often endophytic growth and less papillary configuration</td>
</tr>
<tr>
<td><strong>II. Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td>Well-differentiated with keratin and parakeratin pearl formation</td>
<td>Various degrees in differentiation, including well, moderate, and poor</td>
</tr>
<tr>
<td>Appearance</td>
<td>Exophytic verrucous configuration</td>
<td>Less papillary growth</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>Less or no</td>
<td>Yes</td>
</tr>
<tr>
<td>Breakage of basement membrane</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>III. Prognosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-year survival rate</td>
<td>75%</td>
<td>40-50%</td>
</tr>
<tr>
<td>Metastasis, regional and distant</td>
<td>Never or seldom</td>
<td>Often</td>
</tr>
</tbody>
</table>

\(^1\) FOM, Floor of mouth.
Despite these differences, a close relationship also exists between VC and SCC. VH and VC often co-exist with dysplasia and SCC, and VH and VC have been reported to progress into SCC (Shear and Pindborg, 1980; Slootweg and Muller, 1983).

The difference between VH and VC is not as striking as between dysplasia and SCC. Clinically VH, similarly to VC, presents as verrucous lesions although the verrucous corrugations are less striking than those of VC. Histologically VH again is similar to VC and presents as verrucous lesions. However, VH is exophytic and the verrucous lesion is superficial in relation to the adjacent normal epithelium (Shear and Pindborg, 1980, Figure I-3a). On the other hand, VC grows both exophytically and endophytically relative to the adjacent normal epithelium (Figure I-3b). Although dysplastic changes are either absent or minimal in VH, the malignant transformation rate of VH to VC is over 70%, which is much higher than that of the premalignant lesions with dysplasia (16-50%) (Murrah and Batsakis, 1994; Arendorf and Aldred, 1982).

**Figure 3.** a, Photomicrograph of a VH. The verrucous growth is exophytic (solid arrow indicates the normal location of the oral epithelium). b, Photomicrograph of a VC. Solid arrow shows that tumor growth is mainly endophytic with large bulbous rete ridges pushing downward relative to the normal location of the overlying oral epithelium (hollow arrow).
I.1.3. Grading and staging of oral cancers

There is essentially no difference at the microscopic level between SCC arising in the oral cavity and those seen at other sites. There are three levels of differentiation for oral cancers: Grade 1, well differentiated; Grade 2, moderately differentiated; and Grade 3, poorly differentiated (Pindborg et al., WHO, 1997). This grading is generally based on the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity. Despite the widespread conventional use of this classification, there appears to be only a limited relationship of this parameter with either outcome or response to treatment.

Widely accepted staging system, the tumor-node-metastasis (TNM) classification system, is summarized for oral cancer in Table 4 (Hermanek and Sobin, 1992; AJCC, 1993). This system evaluates 3 basic clinical features: the size (in centimeter) of the primary tumor; the presence, number, size, and spread (unilateral or bilateral) to the local lymph nodes; and the presence or absence of distant metastasis. The individual clinical parameters in the TNM classification system are grouped to determine the appropriate disease stage (Table 5). Generally, stages are ranked numerically from 0 to IV with decreasing prognosis (Lindberg, 1972; Silverman and Gorsky, 1990). For patients with stage I, the 3-year survival rate is 70-85%, while patients with stage II have 50-60% survival, and those with T3 and T4 lesions have only a 15-20% survival rate (Krupala and Gianoli, 1993; Epstein, 1994).
### Table 4. TNM Staging System for oral cancer

<table>
<thead>
<tr>
<th>Primary Tumor (T)</th>
<th>Regional lymph node (N)</th>
<th>Distant Metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis Carcinoma <em>in situ</em></td>
<td>NO No regional lymph node metastasis</td>
<td>MO No distant metastasis</td>
</tr>
<tr>
<td>T1 Tumor 2 cm or less in greatest dimension</td>
<td>N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension</td>
<td>M1 Distant metastasis</td>
</tr>
<tr>
<td>T2 Tumor more than 2 cm but not more than 4 cm in greatest dimension</td>
<td>N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension</td>
<td></td>
</tr>
<tr>
<td>T3 Tumor more than 4 cm in greatest dimension</td>
<td>N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension</td>
<td></td>
</tr>
<tr>
<td>T4 Tumor invades adjacent structures</td>
<td>N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N3 Metastasis in a lymph node more than 6 cm in greatest dimension</td>
<td></td>
</tr>
</tbody>
</table>

*Adapted from AJCC, 1993.*

### Table 5. TNM clinical stage grouping

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumor Size</th>
<th>Nodal Involvement</th>
<th>Distant Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>T1-T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>T4</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N2-N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>

*Adapted from AJCC, 1993.*
1.1.4. Etiology of oral cancer

Understanding causative factors is essential for prevention strategies to be developed. It is generally accepted that oral SCC has a multifaceted etiology and that it involves a combination of environmental and genetic risk factors. The potential roles of tobacco and alcohol consumption in addition to human papilloma virus (HPV) infection and genetic susceptibility will be each examined below as they impact on studies in this thesis.

1.1.3.1. Tobacco and oral cancer

Tobacco use and alcohol consumption are widely considered to be the major risk factors for development of oral cancer (Blot et al., 1988). Although other lifestyle and environmental factors also have been identified as risk factors for oral cancer (e.g. mouthwash use, poor dentures), tobacco use remains the single most important and preventable cause (Andre et al 1995; Bundgaard et al., 1994; Paterson et al., 1996; Vokes et al., 1993). Reports by the United States Public Health Service have clearly established a direct causal relationship between cigarette smoking and oral cancer (1982, 1989). Smoking is responsible for more than 90% of oral cancer deaths in males (Cinciripini and McClure, 1998). All forms of tobacco - cigarettes, pipes, cigars, and snuff and chewing tobacco - have been implicated in the development of oral cancers (Spitz and Newell, 1987; US DHEW, 1973; Winn et al., 1981). For example, the incidence of HNSCC is as high as 40% in the Far East and India (Saranath et al., 1993), where usage of smokeless tobacco is the more common habit, frequently starting at a young age. Smoking of bedi (tobacco rolled in a temburni leaf) and reverse-smoking are also prevalent in
these regions. In addition, patients developing oral cancer often have dietary deficiencies for nutrients such as vitamin A and C, iron and other trace elements (Paterson et al., 1996). While nutritional deficiency is not carcinogenic, deficiency of some factors may result in a decreased protection against development of cancer.

Cancer causation by tobacco usage is dose- and time- dependent: longer exposure and larger consumption of tobacco increase the risk of developing cancer (US DHHS, 1982; Winn et al., 1981). The risk of cancer development is particularly high for individuals who began smoking before age 18 and sustained the habit for 35 years or longer (Blot et al., 1988; Bundgaard et al., 1994). Cessation of cigarette smoking is associated with a 50% reduction of risk of developing oral cancer within 3 to 5 years (Samet, 1992) and a return to a normal level of risk within 10 years (Blot et al., 1988). Tobacco usage is strongly associated with both oral SCC and VC, although some believe VC is more commonly seen in those using smokeless tobacco (Rosenfeld and Callaway, 1963; Brown et al., 1965).

Tobacco contains numerous carcinogens, such as aromatic hydrocarbons and nitrosamines. Chemical analysis reveals that smoke from a single cigarette is composed of over 4,000 different constituents, including some that are pharmacologically active, toxic, mutagenic, or carcinogenic (US DHEW, 1979). For example, polynuclear aromatic hydrocarbons (PAHs) in tobacco smoke have been implicated extensively in oral carcinogenesis, and 4-(methylnitrosamino)-1,3-pyridyl-1-butanone (NNK) and \( N^\prime - \) nitrosonornicotine (NNN), which are found in both smokeless tobacco and tobacco smoke, likely play a major etiological role in oral cancer as well (Brunneman et al., 1982; Hoffman et al., 1995).
1.1.3.2. Alcohol and oral cancer

The risk associated with alcohol consumption is not as well understood as that of tobacco. It is not clear whether alcohol by itself is carcinogenic, since heavy drinkers tend to smoke as well. Another problem is that alcoholics are not infrequently malnourished and chronic alcohol consumption may affect the liver's ability to deal with toxic or potentially carcinogenic compounds (Harris et al., 1996). Furthermore, the assessment of alcohol intake is inherently imprecise because of a bias toward underreporting and the often episodic nature of usage. Thus, it is hard for a patient to estimate "average" use. All three forms of alcohol (beer, hard liquor, and wine) have been associated with oral cancer, although hard liquor and beer have a higher associated risk (Kabat and Wynder, 1989; Mashberg et al., 1981).

Indeed most studies suggest that alcohol acts as a co-carcinogen and that there is a synergistic effect of tobacco and alcohol (Andre et al., 1995; Ng et al., 1993). One possible mechanism proposed for the co-carcinogenic effect of alcohol is that chronic ethanol consumption will cause oral mucosal atrophy with associated hyper-regeneration, which in turn may result in an enhanced susceptibility of the mucosal epithelium toward other carcinogens (Maier et al., 1994; Valentine et al., 1985). Another possibility is that alcohol serves as a solvent for carcinogens, increasing their penetration into the mucosa (McCoy and Wynder, 1979; Simanowski et al., 1995). Co-carcinogenic activity may also be due to liver damage caused by chronic alcohol consumption, which could reduce clearance of carcinogens from the body. It has also been suggested that alcohol may have an effect on DNA repair mechanisms (Mufti et al., 1988). Hsu and Furlong (1991) found that ethanol may temporarily inhibit DNA repair.

Whatever the mechanism involved, there is no doubt that the combined usage of tobacco products and alcohol markedly increases the risk of cancer development (Spitz, 1994). This
increase may be by as much as 50% to 100% over the rates observed in nondrinking smokers or nonsmoking drinkers (Newcomb and Carbone, 1992). An additional effect of these habits is that they appear to also be involved in the risk of development of second cancers. In 1994, a nested cohort study was designed to evaluate the role of tobacco and alcohol on the development of second cancers following oral and pharyngeal cancers (Day et al., 1994). That study showed that tobacco and alcohol each contributed to risk of second cancers, with the effects of smoking more pronounced than those of alcohol. Current smokers showed a fourfold increased risk for second cancers compared to nonsmokers and former smokers. Risk was significantly reduced, however, five years after smoking cessation.

1.1.3.3. HPV and Oral Cancer

The causes of oral SCC have not been fully elucidated, although the roles of tobacco and alcohol as etiologies of oral SCC have been firmly established. Microorganisms, particularly oncogenic viruses, have been suspected of playing a role in oral cancer development, but supporting evidence is still weak. Human papillomavirus (HPV) infection is believed to be an essential step in the carcinogenesis of anogenital cancer. There has been increasing interest recently on the role of HPV in oral cancer development.
HPV genome organization

Over the past decade there have been dramatic developments in molecular biology and virology, giving greater insight into the behavior of viruses, identifying new viruses and elucidating different mechanisms of viral carcinogenesis (Cox et al., 1993).

HPV are small DNA viruses that infect squamous epithelia (Moles et al., 1994). The HPV genome consists of about 7900 base pairs of double-stranded circular DNA encapsulated within an icosahedral protein shell (capsid), 55 nm in diameter (Murphy, 1996). DNA regions that encode viral proteins are called 'open reading frames' (ORFs) and are present on only one of the DNA strands. ORF sequences are divided into early (E) and late (L) regions. E regions, designated E1-E7, are expressed soon after infection and encode proteins involved in the induction and regulation of viral DNA replication. L regions, designated L1 and L2, are expressed later in the infection and encode viral capsid proteins (Shroyer and Greer, 1991). From the standpoint of cellular transformation, the L1, E6, and E7 regions are of major importance (Snijders et al., 1996; Figure 4).

Figure 4. Human papillomavirus genome organization

(Adapted from Murphy, 1996)
Currently, the polymerase chain reaction (PCR) technique is the favored technique for classification of HPVs. The DNA of the virus is amplified and sequenced. If the homology of ORFs E6, E7 and L1 is less than 90%, the genome is defined as constituting a new ‘type’ and is numbered in order of discovery. Homology in excess of 90% renders the virus a subtype and homology of more than 98% indicates a variant. Until now, more than 80 HPV types have been identified and fully sequenced; more than 120 putative novel types have been partially characterized (zur Hausen, 2000).

**HPV and cervical cancer**

Uterine cervical cancer represents the second most common malignancy in women worldwide, both in incidence and mortality (Cannistra and Niloff, 1996). Although there are several factors that might be related to the development of this tumor, HPV infection is considered to be the essential initiating event in cervical epithelial transformation (zur Hausen, 1991).

HPV infections are common. Infection by HPV may lead to an asymptomatic condition, or may produce various benign and malignant lesions. Most HPV types are found in benign epithelial proliferations, e.g., anogenital condyloma acuminatum (genital/venereal warts). These are termed ‘low-risk’ HPV (zur Hausen, 1991). A specific subset of HPVs, including HPV types 16, 18, 31, 33 and 39, is detected in up to 99% of anogenital preneoplastic lesion and SCC. These are termed ‘high-risk’ HPVs (zur Hausen, 1991). Viral genomes of this ‘high-risk’ set are usually integrated into host DNA and are transcriptionally active in both tumors and tumor-derived cell lines. Based on this evidence, the International Agency for Research on Cancer (IARC, 1997) has classified HPV 16 and 18 as carcinogenic in humans (Group 1), HPV 31 and
33 as probably carcinogenic in humans (Group 2A) and some of the remaining HPV types as possibly carcinogenic in humans (Groups 2B).

It is generally believed that these viruses are necessary but insufficient in themselves to cause cervical SCC and other anogenital SCC. Only a fraction of HPV infected patients develop cancer and high-risk HPVs are also detected in histologically normal cervical cells (de Villiers et al., 1987), suggesting that high-risk HPV infection may be a co-factor in cervical carcinogenesis. In the relationship of HPV infection and cervical cancer, viral oncogene expression and HPV integration must cooperate with a specific genetic background of the host to initiate the multi-step process of cervical carcinogenesis (zur Hausen, 2000).

Possible mechanism of action of HPV in squamous epithelial carcinogenesis - lessons learned from cervical cancer

The virus infects the basal cells of the epithelium. As these cells proliferate, the viral DNA also proliferates, maintaining a low copy number of episomal viral DNA molecules in the daughter cells. For a variable period of time the virus is latent, with no evidence of infection. There is usually a prolonged lag time of decades between viral exposure and appearance of the cancer. All evidence suggests the malignant progression requires not only the presence of the virus but also cellular mutations. When malignant conversion occurs, the expression of the virus changes (zur Hausen, 1996). Usually, the viral DNA integrates into the host chromosomes in such a way that only the E6 and E7 genes are expressed. There is no longer productive viral DNA synthesis, or synthesis of capsid proteins, and new virus is no longer made. Thus, induction of cancer is a dead end for the viral life cycle.
Evidence in support of a role for HPV in tumor development is strongly dependent upon a determination of whether or not the DNA is integrated into the host DNA. If it is still episomal, there is a question of whether or not its presence is merely a coincidence, rather than causal. In addition, the expression in a tumor of viral RNA that codes for the E7 gene product is taken as a strong indication that the virus is playing an important role in the formation of cervical cancer (Aggelopoulou et al., 1998). Progression of HPV 16 positive tumors appears to involve both amplification (increased copy numbers) and integration of the viral DNA. In contrast, progression of HPV 18 positive tumors seems to be more dependent on the integration of the viral DNA (zur Hausen, 2000).

HPV infection deregulates host cell cycle control via interaction of viral oncoproteins with host cell tumour suppressor gene (TSG) proteins (Alani and Munger, 1998; Boyer et al., 1996; Werness et al., 1990). E6 oncoprotein binds with p53 and triggers its inactivation. E7 interacts with Rb. The proteins coded by p53 and Rb are critical for G1 cell cycle arrest after DNA damage. Loss of this functions is critical for tumor development. In addition, the binding of E6 and E7 with p53 and Rb is associated with cell immortalisation (zur Hausen, 2000; Figure 5).
In oral keratinocytes infected with high risk HPV 16/18, E6 binds and deactivates p53. Following DNA damage, p53 and p21 are not upregulated and hence there is no inhibition of pRb phosphorylation. Phosphorylated pRb releases active E2F transcription factors. High risk HPV 16/18 E7 protein binds hypophosphorylated pRb also causing the release of active E2F transcriptional factors. This, combining with PCNA, stimulates DNA replication and oral keratinocyte division. Thus, high risk HPV infection may result in oral cancer development (Adapted from Sugerman and Shillitoe, 1997).
HPV and oral cancer development

The mucosal epithelium in the oral cavity is histologically and embryologically similar to that of the genital tract and is continuously exposed to various environmental factors such as irritants and microorganisms. Thus, it is reasonable to assume that there are parallels between uterine cervix and the oral cavity with respect to the capacity of HPV to participate in malignant transformation. We cannot neglect the role of HPV in oral carcinogenesis, especially for those without apparent carcinogen exposure (i.e. nonsmokers and nondrinkers) or those with only limited carcinogen exposure (young cancer patients) (Matsuda et al., 1996; Snijders et al., 1996; Woods et al., 1993).

Studies on HPV infection and oral premalignant and malignant lesions show that the most common type of HPV are types 16 and 18. However, there are tremendously diverse results on the frequency of infection (Table 6). Positive and negative correlations have been reported between HPV infection and HNSCC including oral SCC. In addition, some studies showed similar frequency of HPV infection in HNSCC and adjacent normal looking mucosa. The frequency of HPV detection in clinically healthy mucosa in the head and neck region ranges from 0% to 67% (Woods et al., 1993; Yeudall and Campo, 1991), while rates reported for HNSCC including oral SCC range from 0-78% of cases (Palefsky et al., 1995; Shroyer and Greer, 1991; Woods et al., 1993).

In a recent case-control study, HPV presence in the mouth rinse was associated with increased risk of oral or oropharyngeal cancer (OR = 3.7), independent of alcohol and tobacco exposure (Smith et al., 1998). In another comprehensive review of head and neck SCC, McKaig et al. reported that the overall prevalence of HPV positivity assessed by PCR was 34.5% (416 of 1205 tumors) (1998).
Many of the studies quoted in Table 6 are done on HNSCC including oral SCC instead of only oral SCC. A recent study with a large number of fresh head and neck SCC from an esteemed research laboratory (Sidransky’s group) in Johns Hopkins hospital investigated HPV infection in oropharyngeal and oral SCC. While a low rate of HPV was found in oral SCC (12% of cases), a high rate was found for oropharyngeal SCC (62%, Gillison et al., 2000). In Table 6, there was a similar trend: HPV infection in oral SCC was 21% (76/363) in contrast to 26% (232/901) noted in HNSCC which include SCC in other head and neck regions ($p = 0.0449$).
Table 6. Prevalence of HPV infection in oral or head and neck SCCs as determined by PCR

<table>
<thead>
<tr>
<th>Study</th>
<th>Frequency of HPV infection in SCC</th>
<th>HPV type in SCC</th>
<th>Frequency of HPV in normal oral mucosa</th>
<th>HPV type in normal oral mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral SCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shroyer and Greer, 1991</td>
<td>1/10 (10%)^4</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yeudall &amp; Campo, 1991</td>
<td>18/39 (46%)</td>
<td>16, 18</td>
<td>2/25 (8%)^1</td>
<td>18</td>
</tr>
<tr>
<td>Ostwald et al., 1994</td>
<td>16/26 (62%)</td>
<td>6, 11, 16, 18</td>
<td>1/97 (1%)^1</td>
<td>1/26 (4%)^2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7/26 (27%)^3</td>
</tr>
<tr>
<td>Shindohe et al., 1995</td>
<td>8/24 (33%)</td>
<td>16, 18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Haraf et al., 1996</td>
<td>0/14 (0%)</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Snijders et al., 1996</td>
<td>5/25 (20%)</td>
<td>16</td>
<td>7/30 (23%)^2</td>
<td>16</td>
</tr>
<tr>
<td>Gopalakrishnan et al.,1997</td>
<td>3/10 (30%)</td>
<td>16</td>
<td>1/10 (10%)^1</td>
<td>16</td>
</tr>
<tr>
<td>Matzow et al., 1998</td>
<td>1/38 (3%)</td>
<td>Unknown (not 6, 11, 16 or 18)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Smith et al., 1998</td>
<td>14/93 (15%)</td>
<td>16, 18</td>
<td>10/205 (5%)^1</td>
<td></td>
</tr>
<tr>
<td>Gillison et al., 2000</td>
<td>10/84 (12%)</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>76/363 (21%)</td>
<td></td>
<td>14/337 (4%)^1</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>HPV Positive/Total (Percentage)</td>
<td>HPV Detected</td>
<td>HPV Detected</td>
<td>Remarks</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>Watts et al., 1991</td>
<td>16/23 (70%)</td>
<td>6, 11, 16, 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachman et al., 1992</td>
<td>1/30 (10%)</td>
<td>16, 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maden et al., 1992</td>
<td>6/108 (6%)</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holladay and Gerald, 1993</td>
<td>7/37 (19%)</td>
<td>16, 18</td>
<td>1/6 (17%)†</td>
<td>16</td>
</tr>
<tr>
<td>Woods et al., 1993</td>
<td>14/18 (78%)</td>
<td>16, 18, 6, 11</td>
<td>6/9 (67%)‡</td>
<td>6, 11</td>
</tr>
<tr>
<td>Anderson et al., 1994</td>
<td>8/35 (23%)</td>
<td>16, 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palefsky et al., 1995</td>
<td>9/26 (34%)</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cruz et al., 1996</td>
<td>19/35 (43%)</td>
<td>16</td>
<td>0/12 (0%)†‡</td>
<td></td>
</tr>
<tr>
<td>Haraf et al., 1996</td>
<td>10/26 (38%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mao et al., 1996a</td>
<td>12/41 (29%)</td>
<td>16</td>
<td>0/6 (0%)†</td>
<td></td>
</tr>
<tr>
<td>Snijders et al., 1996</td>
<td>13/63 (21%)</td>
<td>16</td>
<td>7/30 (23%)‡</td>
<td>16</td>
</tr>
<tr>
<td>Fouret et al., 1997</td>
<td>12/65 (19%)</td>
<td>16, 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matzow et al., 1998</td>
<td>3/16 (19%)</td>
<td>Unknown</td>
<td>(not 6, 11, 16 or 18)</td>
<td></td>
</tr>
<tr>
<td>Koch et al., 1999</td>
<td>37/163 (18%)</td>
<td>16, 33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand et al., 2000</td>
<td>3/24 (12.5%)</td>
<td>16, 18, 6, 11</td>
<td>0/12 (0%)†‡</td>
<td></td>
</tr>
<tr>
<td>Gillison et al., 2000</td>
<td>62/191 (62%)</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>232/901 (26%)</strong></td>
<td><strong>1/24 (4%)†‡</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Normal appearing mucosa from patients without oral cancer or premalignant lesion;
2 Normal appearing mucosa adjacent to tumors; 3 Normal appearing mucosa distant from tumor;
4 Number of cases positive for HPV/total number studied (percentage)
Few studies of HPV infection on oral premalignant lesions were reported (Mao et al., 1996a,b; Nielsen et al., 1996; Sand et al., 2000). These studies was limited by case number and no conclusive remarks can be made.

Possible reasons for the discrepancy in the detection rate of HPV DNA

1. **Assay sensitivity.** This is the major reason for the discrepancy in results between studies. Assays considered to be of low sensitivity include those that involve immunohistochemical analysis detected with immunoperoxidase or immunofluorescence and *in situ* hybridization for HPV-DNA. These assays are estimated to have a requirement for detection limit of >10 copies of viral DNA per cell (de Villiers et al., 1989). Assays considered to be of moderate sensitivity include Southern blot, dot blot and reverse blot hybridization. An assay considered to be of high sensitivity uses the polymerase chain reaction (PCR), which can detect < 1 copy of viral DNA per cell (Miller and White, 1996).

2. **The method of specimen storage.** HPV DNA is detected significantly more often in fresh or frozen sections of oral SCC as compared with paraffin-embedded tissue (58% of fresh samples versus 17% of paraffin, Ostwald et al., 1994). This is particularly true if the PCR product is large (>200bp; Karlsen et al., 1994).

3. **Physical status of HPV genome.** With integration of viral DNA into the cell genome, deletion of parts of genome could occur. This has shown to be relatively common in malignant cervical cancer (zur Hausen and Schneider, 1987). When the viral genome
integrates, deletions occur in the L1 ORF, to which most consensus primers are directed. If all HPV DNA is integrated, this deletion leads to false negative PCR results. However, it should be noted, that although HPV DNA integration is viewed as an integral component to the progression of cervical lesions, it appears to be a less frequent event in oral cancers (de Villiers et al., 1987; Maitland et al., 1987; Kashima et al., 1990; Watts et al., 1991; Yeudall and Campo, 1991).

4. **'Hit and Run' theory.** A transient role of virus in the induction of carcinomas cannot be ruled out (zur Hausen, 1982).

5. **Focal nature of infection.** Infection of HPV may occur focally in a lesion and negative results could stem from not including the positive areas in the analysis (Snijders et al., 1997; Ostwald et al., 1994).

**HPV and verrucous lesion**

There has been a strong suspicion that HPV plays a critical role in the pathogenesis of VCs. Such suspicion is mainly based on the clinical and histological features of the lesions. The verrucous or papillary configuration of the verrucous lesions is strongly suggestive of HPV etiology. The appearance of these oral lesions resembles that of oral squamous papilloma, verruca vulgaris and venereal warts, all of which are accepted to be HPV induced (Fornatora et al., 1996; Young and Min, 1991). Likewise, histologically, verrucous lesions contain cytological alterations (e.g. presence of koilocytes) that are characteristic of lesions with HPV infection. In one study, 15 out of 17 cases showed an epithelial nuclear morphology similar to verruca...
vulgaris and venereal warts, where HPV infection is accepted (Eisenberg et al., 1985). However, these results only provide indirect evidence of the involvement of HPV. Unfortunately, more direct studies, in which samples were assayed for the presence of HPV-DNA have not resolved this issue. Again, as in other oral lesions, results obtained from the verrucous lesions were controversial. Infection rates for verrucous lesions have varied from 0% to 89% (Gopalakrishnan et al., 1997; Noble-Topham et al., 1993; Palefsky et al., 1995; Shroyer and Greer, 1991; Shroyer et al., 1993; see summary in Table 7).

Table 7. Prevalence of HPV genotypes in verrucous lesions as determined by PCR

<table>
<thead>
<tr>
<th>Study</th>
<th>Study group</th>
<th>Frequency of HPV in oral lesions</th>
<th>HPV types</th>
<th>HPV in normal oral mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shroyer et al., 1991</td>
<td>VH</td>
<td>4/14 (29%)</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VC</td>
<td>0/3 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shroyer et al., 1993</td>
<td>VC</td>
<td>7/17 (41%)</td>
<td>6/11</td>
<td>-</td>
</tr>
<tr>
<td>Noble-Topham et al.,</td>
<td>VC</td>
<td>12/25 (48%)</td>
<td>16,18,6,11</td>
<td>-</td>
</tr>
<tr>
<td>1993</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palefsky et al., 1995</td>
<td>PVL</td>
<td>8/9 (89%)</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Gopalakrishnan et al.,</td>
<td>PVL</td>
<td>2/10 (20%)</td>
<td>16; 18</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>1997</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Number of cases positive for HPV/total number studied (percentage)

2 PVL: Proliferative verrucous leukoplakia
**HPV and immunocompromised patients**

The number of immunocompromised patients is increasing as a consequence of the growing number of HIV-infected patients as well as the aging population. In addition, the widespread iatrogenic immunosuppression that is associated with transplantation of various organs leads to another group of immunosuppressed patients. The immunosuppression could significantly alter the susceptibility of such individuals to infection, including oncogenic virus HPV. It could also have an impact on immunosuppression on outgrowth of premalignant lesions.

HPV infection has been compared in skin SCCs from renal transplant recipients and skin cancer patients that have had no transplantation. A study found that HPV-related sequences were present much more frequently in cancers from immunosuppressed patients (13 of 20 cases compared to 8 of 26 cases, $p = 0.03$, Shamanin *et al.*, 1996). It is worth noticing that most of the viruses identified in these transplant patients were novel types, different from the common ‘high-risk’ types of HPV found in cervical cancer. An additional finding was that transplant patients were more likely to have persistent cutaneous HPV infection in benign and premalignant skin lesions (Berkhout *et al.*, 2000).

A study on oral dysplasia and SCC found that HPV16 E6 was roughly 10 times more likely to be expressed in tumors from immunocompromised patients than in those of immunocompetent patients ($p = 0.0008$; Al-Bakkal *et al.*, 1999). These data suggested that HPV might play a role in the development of oral cancer in association with immunosuppression.
1.1.3.4. Genetic susceptibility to oral cancer

For any given level of exposure to carcinogens, only a proportion of exposed individuals will develop cancer. This suggests that some individuals are resistant to the exposure. On the other hand, there is an emerging population of cancer patients who lack exposure to known agents (Fouret et al., 1997; Koch et al., 1999; Wey et al., 1987). In the latter case, an intrinsic susceptibility to environmental genotoxic exposures has been suggested as playing a role in the carcinogenesis.

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

I.2. Unresolved clinical and histopathological issues that are the focus of this thesis

I.2.1. Difficulties associated with the diagnosis of high-risk oral premalignant lesions

Oral premalignant lesions (OPL), which typically appear as leukoplakia, are a heterogeneous group of lesions with varying risk of malignant transformation. Currently, histological criteria (the presence and degree of dysplasia) represent the gold standard for judging this risk, although other factors are also used, such as clinical appearance, site and size of the lesions (Schepman and van der Waal, 1995; van der Waal et al., 2000). However, the above criteria have a limited ability to predict cancer risk of OPL.

The histological progression model has a better predictive value for high-grade preinvasive lesions (severe dysplasia and CIS) which are believed by many to have a much higher possibility of progression into invasive lesions than low-grade lesions, including hyperplasia without dysplasia and those with low-grade dysplasia (mild and moderate dysplasias) (Banoczy and Csiba, 1976; Schepman et al., 1998). Some even believe that oral CIS will inevitably become cancer, if left untreated (Hayward, 1977). As a result, high-grade
preinvasive lesions are generally treated aggressively, and the histological diagnosis serves as the stimulus for that aggressive treatment.

In contrast, the histological progression model has a poor predictive value for lesions without dysplasia or with mild or moderate dysplasia. At this stage, the degree of dysplasia provides little indication of risk of progression to cancer (Mincer et al., 1972; Schepman et al., 1998; Silverman et al., 1984). Indeed, the majority of these lesions do not progress, they either remain static or on occasion regress. Unfortunately, as a group, such lesions account for the majority of leukoplakia. For example, in a study with a large number of cases by Waldron and Shafer (1975), these lesions accounted for 92% of leukoplakias: percentage of cases with hyperplasia without dysplasia, 80%; low-grade dysplasia, 12%; high-grade dysplasia, 4.5% and SCC, 3%. Aggressive treatment does not seem to be justified for the majority of these lesions, in terms of risk, side effects and cost. New approaches that could assist the clinician in differentiating the small percentage of progressing low-grade lesions from the majority of non-progressing low-grade lesions are highly desirable.

Another barrier to the use of histologic diagnosis to select low-grade lesions for treatment is the subjective nature of these diagnoses in early stages. Discrepancies in diagnoses are frequently found between pathologists at this stage (Abbey et al., 1995; Lumerman et al., 1995; Pindborg et al., 1985). The development of more objective and reproducible measures, such as genetic markers, in risk prediction for oral premalignant lesions would again be desirable.

Would the understanding of the molecular changes in these early lesions improve our ability to predict their cancer risk? This is one question that this thesis has begun to address.
1.2.2. Problems in management of oral premalignant lesions

As a result of the above problems, treatment of OPL varies widely and has been a topic for heated debate (Schepman et al., 1998). As shown in Table 8, the majority of literature suggest surgical removal of leukoplakia could result in a decreased cancer transformation rate compared to 'wait and see' (Bancozy and Cisba, 1976; Chiesa et al., 1993; \( p = 0.0001 \)), there are studies showing that removal has no effect on lowering the cancer risk (Mincer et al., 1972; Schepman et al., 1998; Silverman et al., 1984; Sato et al., 1999; Einhorn and Wersall, 1967).

For example, a report by Banoczy and Csiba strongly supports removal. In that study, only 1 of 45 patients whose leukoplakias were surgically removed went on to develop invasive SCC, compared to 8 of 22 patients in which the lesion received no treatment \( (p = 0.0003; \) Bancozy and Cisba, 1976). In a second study, Chiesa analyzed 167 consecutive patients with leukoplakias that received laser therapy: only five patients (3%) developed oral cancer within 5 years of the operation (Chiesa et al., 1993). In contrast to the results of these 2 studies, Mincer found that 2 of 22 dysplastic lesions developed cancer after surgical removal compared with 3 out of 20 cases in which a "wait and see" policy was used \( (p = 0.656; \) Mincer et al., 1972, also see Table 8).

These conflicting results in the literature have led to a situation where there is no consensus on treatment for early oral lesions. Among the different management regimens in use are a wide range of activities from "wait and watch" to chemical therapies with bleomycin, anti-inflammatory agents or vitamins, to surgical removal by scalpel, laser, cryoprobe or electrosurgery.

The lack of consensus on treatment is also reflected in the fact that even among clinicians who agree that leukoplakia should be removed, there is no concurrence on an appropriate margin of clinically normal mucosa to be removed along with the lesion. A large percentage of
clinicians simply remove the clinically visible lesion without margin, while others would remove these lesions with a small, normal-looking margin (e.g., 2-4 mm). Only rarely do surgeons remove an OPL with a wide margin. The high recurrence rates for surgical excision (12 to 25%; Chiesa et al., 1993; Mincer et al., 1972) are probably a result of insufficient removal.

This study evaluated some of the local practices that Vancouver clinicians use in treating OPL. It also explored the use of molecular techniques to evaluate margins for completeness of removal.

Table 8. Summary of treatment effect on malignant transformation rate of OPL

<table>
<thead>
<tr>
<th>Author</th>
<th>Study group</th>
<th>Follow up (yr)</th>
<th>Wait &amp; See: Rate of cancer progression</th>
<th>Excision: Rate of cancer progression</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mincer et al., 1972</td>
<td>Moderate/severe dysplasia, CIS</td>
<td>8</td>
<td>2/22 (9%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3/20 (15%)</td>
<td>0.6560</td>
</tr>
<tr>
<td>Banoczy &amp; Csiba, 1976</td>
<td>Dysplasia</td>
<td>20 (mean 6.3)</td>
<td>8/22 (36%)</td>
<td>1/45 (2%)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Lumerman et al., 1995</td>
<td>Dysplasia</td>
<td>20</td>
<td>14/91 (15.4%)</td>
<td>4/65 (6%)</td>
<td>0.0823</td>
</tr>
<tr>
<td>Pindborg et al., 1977</td>
<td>Dysplasia</td>
<td>NA</td>
<td>4/47 (8.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vedtofte et al., 1987</td>
<td>Dysplasia</td>
<td>1-5 (mean 3.9)</td>
<td></td>
<td>3/61 (5%)</td>
<td></td>
</tr>
<tr>
<td>Chiesa et al., 1993</td>
<td>Leukoplakia</td>
<td>4.5</td>
<td></td>
<td>5/167 (3%)</td>
<td></td>
</tr>
<tr>
<td>Schepman et al., 1998</td>
<td>Leukoplakia</td>
<td>0.5-17.5 (mean 2.4)</td>
<td></td>
<td>20/166 (12%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>48/348 (14%)</strong></td>
<td><strong>16/358 (4.5%)</strong></td>
<td>0.0001</td>
</tr>
</tbody>
</table>

<sup>1</sup> number of cases with later progression/number of total cases studied (% of cases in parentheses)
1.2.3. Lack of information regarding the relationship of VH and VC with dysplasia and SCC

This problem was initially discussed in Section I.1.2.5. (Histopathology and histopathological progression model of verrucous carcinoma). Verrucous lesions (VH and VC) differ from dysplasia and SCC clinically, histologically and prognostically (Table 3) and may have their own histological progression pathways (see Figure 2 and Figure 3). It is important to note that these pathways on occasion may interact. For example, oral dysplasia and SCC are often found in patients with oral VH or VC, either concurrently or at a different time. VH or VC can also become dysplastic and on occasion, develop further into invasive SCC. What is the relationships between these two clinical pathways? Is there an underlying molecular basis that controls them? These are questions that this thesis has begun to address.

1.2.4. Difficulties associated with the diagnosis of verrucous lesions

Both VH and VC frequently present diagnostic challenges for pathologists. For conventional oral premalignant lesions, the "gold" standard used in judging malignant risk of epithelial lesions is the presence of dysplasia both cytologically and architecturally in biopsies. The higher degree of dysplasia present, the higher malignant risk presumed. For SCC, an additional parameter is ascertained, the presence of invasion, that is, islands of dysplastic epithelium that have broken the basement membrane and spread into connective tissue stroma. However, as discussed above, both VH and VC show minimal or no dysplasia and show no breakage of basement membrane. For these reasons, VH and VC are frequently misdiagnosed as
reactive hyperplastic lesions, such as frictional hyperkeratosis, which can have slightly verrucous surface, or as HPV-induced reactive lesions (e.g. venereal warts), which are typically verrucous and can contain a certain degree of 'dysplastic' changes that are related to virus infection (Adkins and Monsour, 1976; Demian et al., 1973; Dunlap and Barker, 1985; Eversole and Papanicolaou, 1983; Ferlito et al., 1998; Rink, 1991; Shear and Pindborg, 1980). Such diagnostic difficulties are evident in the literature with the extent of the problem indicated by the need for multiple biopsies (2 to 7 on average) to establish the diagnosis (Biller et al., 1971; Maurizi et al., 1996). Misdiagnosis of VH or VC as reactive hyperplastic lesions carries grave consequences as a delay in the right treatment can result in progression of these verrucous lesions to SCCs which has a much poorer prognosis (Ferlito et al., 1998).

Can we use molecular tools to facilitate the differential diagnosis of these lesions? *This was a question that was addressed in this thesis.*

1.2.5. **Unresolved issues involving etiology and pathogenesis of oral SCCs in patients with previous history of organ transplantation**

1.2.5.1. **Increased incidence of oral SCCs in post-transplant patients**

Transplantation is becoming an increasingly common procedure. Bone marrow transplantation is currently a standard procedure used in treatment of leukemias. There is also an increasing number of patients that receive transplants to replace dysfunctional organs, primarily kidneys, and at a much lower frequency, lung and heart. All of these patients require immunosuppressive therapy to prevent rejection of the transplant. There is growing concern that
a possible late consequence of this immunosuppression is an increase in other diseases, including cancer. For leukemia patients, there is a further risk of new solid cancers that is associated with the high-dose radiation or chemotherapy used to destroy the patient's own bone marrow cells.

In 1997, Curtis and co-workers published a study of 19,229 patients who had received bone marrow transplants over a period of 28 years at 235 centers (1997). Transplant recipients had a significantly elevated risk of formation of new solid cancers compared with the general population (number of observed cases, 80; ratio of observed to expected cases, 2.7; p<0.001). The relative risk was significantly elevated for bone (13.4) and oral cancer (11.1) (Curtis et al., 1997). The risk was higher in patients who were younger at the time of transplantation and among patients that received higher doses of total body irradiation (TBI). Chronic graft-versus-host disease and male sex were strongly linked with an excess risk of SCC in oral cavity and skin. Eight percent of transplant patients had dysplastic lesions of the vermilion border of the lip and 1% had malignant lesions on the lip. In contrast, neither of these lesions was found in the control group (King et al., 1995; also see Table 9).
<table>
<thead>
<tr>
<th>Study</th>
<th>Type of transplantation</th>
<th>Number of patients</th>
<th>Total # of cases developing cancer</th>
<th>Number of oral cancers¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harris and Penn, 1981</td>
<td>Organ</td>
<td>NA</td>
<td>1023</td>
<td>11</td>
</tr>
<tr>
<td>Witherspoon et al., 1989</td>
<td>BMT</td>
<td>2,246</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Curtis et al., 1997</td>
<td>BMT</td>
<td>19,229</td>
<td>80</td>
<td>14</td>
</tr>
<tr>
<td>Socie et al., 1998a</td>
<td>BMT</td>
<td>289</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Duvoux et al., 1999</td>
<td>Liver</td>
<td>NA</td>
<td>90</td>
<td>11</td>
</tr>
</tbody>
</table>

¹This number includes only oral cavity and excludes cases that involve the lip.

### 1.2.5.2. Possible mechanisms for increased oral SCCS in transplant recipients

The mechanisms underlying this increase in cancer risk remain unclear. Defective immune function is suspected to be a major contributing factor. Immunosuppressive treatment is invariably given to people who have received solid organ transplant to avoid organ rejection, and frequently given to people who have received bone marrow transplant (BMT) to alleviate graft-versus-host disease (GVHD), a transplant bone marrow induced immunoreaction against host (Curtis et al., 1997; Deeg et al., 1996; Socie et al., 1998a). Immune defects resulting from these treatments could render the host cancer prone either by faulty immunosurveillance of genetically abnormal cells or by increased infection with microorganisms such as oncogenic viruses (Kinlen 1996). In addition, total body irradiation and chemotherapy treatment for the primary bone...
marrow disease in people received BMT could result in DNA damage which in turn could contribute to the cancer increase (Thompson et al., 1994; Day & Boice, 1983; Hay et al., 1984; Curtis et al., 1997). These possibilities are discussed below.

1. **Effects of immunosuppression treatment.** Severe immunosuppression such as that seen in organ transplant patients or in people with AIDS is known to be associated with an increased incidence of cancer, including oral SCCs. Several hypotheses have been proposed for the increase: (1) an impact of the therapy on the immunosurveillance mechanism that normally acts to prevent the outgrowth of premalignant or malignant cells; (2) an increase in susceptibility to infection by microorganisms including oncogenic virus and candidiasis; and (3) a possible carcinogenic effect of specific immunosuppressing drugs.

   There are controversies on how to handle immunosuppressive therapy in transplant recipients who have developed cancer. Some clinicians choose to reduce or even stop immunosuppression treatment in the hope that the patient’s immune system might recover and help to destroy the cancer cells. However, there have been no studies showing regression of the cancer with reduction or cessation of immunosuppressing treatment in these patients. Many clinicians hence do not modify their treatment with the belief that once the cancer is formed, it will continue to grow regardless whether the immunosuppression drugs are withdrawn. Furthermore, reduction and cessation of the drug entail a possible risk of organ rejections.

2. **Oncogenic viruses.** Immunosuppressed patients are predisposed to infections with potentially oncogenic viruses, such as human papillomavirus (HPV), Epstein Barr virus
(EBV), human hepatitis virus (HHV), and possibly hepatitis C virus (HCV) (Schmidt-Westhausen et al., 1993; Naraqi et al., 1977). These viruses could play a role in the pathogenesis of post-transplant cancer. For example, there is accumulating evidence that implicates HPV in the pathogenesis of skin cancer in renal transplant recipients. A high frequency of HPV-DNA have been reported for skin precursor lesions and tumors among renal transplant recipients.

3. **Chronic GVHD.** Several case report studies have produced data that suggest an increased rate of formation of oral SCCs at a location previously involved with chronic GVHD (Deeg et al., 1996; Socie et al., 1998b). The association of chronic GVHD with posttransplant oral SCCs topographically is possibly a result of both systemic and local factors. On one hand, systemic factor could make the patient more susceptible to cancer in general, and these factors include immunosuppression as a result of immunosuppressive medication for those more symptomatic GVHD and carcinogenic effects of immunosuppressive medications. For example, a recent study of 700 patients with aplastic anemia that received bone marrow transplantation showed that the incidence of solid tumors, predominantly tumors of the oral cavity and skin, was significantly increased after the administration of azathioprine for chronic GVHD (Deeg et al., 1996). On the other hand, chronic GVHD could create local factors that subject the areas with GVHD more susceptible to systemic factors. GVHD is a type of lichenoid mucositis (a chronic mucositis resulted from a cell-mediated local immunological process). Focal inflammation would frequently cause ulceration in the region which could provide a better soil for infection such as by oncogenic viruses. Inflammation increases local cell proliferation which could render the area more sensitive to environmental carcinogenic
effects. The cell-mediated immunological process could also generate focal cell damage hence contributing to the regional carcinogenesis. One study showed that all oral SCCs post BMT demonstrated papillomatous lesions in the cancer area as well as local chronic GVHD (a dense lymphocytic infiltrate) (Socie et al., 1998b). The result is in support of the hypothesis that chronic GVHD provide a better soil for infection by HPV.

4. Effects of total body irradiation. Total body irradiation at the doses typically given to transplant recipients would be fatal without bone marrow transplantation. However, patients who survive high-dose radiation therapy have three to four times increased risk of new cancer formation, with a long latent period, compared with those who had transplants but without irradiation. In a study by Curtis et al. (1997), patients with total body irradiation and BMT showed the following cancers: cutaneous melanomas and SCCs, oral SCCs, cancers of liver, brain or other parts of the central nervous system. In multivariate analyses, higher does of total body irradiation, chronic GVHD, and male sex, were strongly linked with an excess risk of oral and skin SCCs.

One of the goals of this thesis was to obtain a better understanding of the pathogenesis of oral SCC in post-transplant patients. The approach used was to investigate the genetic pathway of SCC induction in these tumors.

1.2.6. Rationale for use of molecular markers to study aforementioned histological and clinicopathological issues
A new approach is needed to study the above issues. This thesis employed recent front-edge molecular techniques to address some of the above questions and problems, in the hope of developing useful approaches to reduce both morbidity and mortality of oral cancer. The studies described in this thesis all focus on clinically relevant issues and the development of approaches that can directly impact on the management of oral premalignant and malignant lesions.

I.3. Critical genetic changes during oral carcinogenesis

The contemporary view of cancer is that a malignancy arises from the transformation of genetic material in a normal cell, followed by successive mutations, ultimately leading to the uncontrolled proliferation of progenitor cells (Bishop, 1991; Vogelstein, 1992). These genetic alterations occur in a multistep fashion in which a cell accumulates many genetic changes, some of which disrupt the balance of normal cell growth and lead to the malignant phenotype. This process is characterized by the evolution of clonal cell populations that possess growth advantage over other cells (Figure 6). Such clones would be expect to persist and probably continue along the neoplastic progression pathway (Fearon and Vogelstein, 1990; Sidransky et al., 1993a).
Two of the most important gene classes involved in carcinogenesis are oncogenes and tumor suppressor genes (TSGs) (Bishop, 1991; Vogelstein, 1992). These two groups of genes act antagonistically to regulate cell growth and differentiation.

I.3.1. Oncogenes and tumor suppressor genes in cancer development

Oncogenes are derived from activation of normal cellular proto-oncogenes. Usually, these genes are positive regulators of (or act to stimulate) critical cellular processes such as cell proliferation. A number of mechanisms have been described in activating these proto-oncogenes
including point mutations, gene amplification and chromosome translocation. This resulting mutation is autosomal dominant meaning that only one of the two gene copies has to be mutated for an effect to be observed. Approximately 50 different oncogenes have been identified. Most of them code for proteins that function as growth factors, growth factor receptors, cytoplasmic second messengers, and regulators of gene transcription.

In contrast to oncogenes, tumor suppressor genes code for proteins that are negative regulators of (or act to block) critical cellular processes such as cell proliferation. The functions of TSGs must be lost in order for tumorigenesis to occur. According to Knudson’s hypothesis (1985), both copies of a tumor suppressor gene have to be inactivated for its protective function to be lost in a cell. Thus mutations to TSGs are said to be autosomal recessive. 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.

A rather simplistic analogy can be made between the functions of these classes of genes in a cell and driving a car (Jordan and Daley, 1997). 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.
I.3.2. **Oncogenes and tumor suppressor genes in oral premalignant and malignant lesions**

Very few oncogenes, \( ras \), \( myc \), \( erbB-1 \) (epidermal growth factor receptor), \( erbB-2 \), \( bcl-1 \), and \( int-2/cyclin-D1 \), have been identified in head and neck cancers (Kiaris et al., 1995; Lese et al., 1995; Saranath et al., 1993; Warnakulasuriya et al., 1992). Our knowledge about 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, 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; 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 due 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 the 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 1999a; 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., 1996c; Mao, 1998; Todd et al., 1995; Uzawa et al., 1994; Waber et al., 1996).

Recent advancement in the techniques of molecular analysis 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 I used in my research in this thesis. Analysis of p53 mutation was also performed. This is one of the most common tumor suppressor genes. These two techniques will be detailed in other sections.

1.3.3. Loss of heterozygosity (LOH) studies using microsatellite analysis

LOH, loss of heterozygosity, is defined as a loss of genomic material (as small as a few thousand nucleotides to as large as a whole chromosome) in one of a pair of chromosomes. The LOH assay is designed to assess polymorphic chromosomal regions that map close to or within putative or known 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 inactivate is required to the remaining allele before expression of the phenotype occurs (Knudson, 1985).
Two methods are available for the study of LOH: restriction fragment length polymorphism (RFLP) analysis and microsatellite analysis. This thesis employed microsatellite analysis for two reasons. First, microsatellite repeat markers are highly polymorphic and well-distributed throughout the human genome. They show levels of heterozygosity between 30-80%, significantly above the level observed with the RFLP analysis based on base substitutions at endonuclease recognition sites. Second, this [$\alpha$-p32] end-labeled PCR-based approach is much more sensitive than the RFLP analysis and requires only a small amount of DNA (5 nanograms or less per reaction). The high sensitivity of the technique is important to these studies because the lesions being examined are frequently small.

Microsatellites contain runs of short and tandemly repeated sequences of di-, tri-, or tetranucleotides, such as –GTGTGT-, –GTAGTAGTA-, or –GTACGTACGTA-. 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 each individual typically containing a different number of copies (generally 4 to 40) of the repeat at each particular locus (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 may be also 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).

1.3.4. The value of LOH studies in cancer research

Information obtained from LOH studies has dual merit. The finding of frequently lost regions during cancer development can lead to discovery of new TSGs. Such was the case for several important TSGs: \textit{Rb}, \textit{MEN1}, \textit{APC}, \textit{NFI}, \textit{NFII}, and \textit{BRCA2} (Ah-See et al., 1994; Fearon, 1997). 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 (Califano et al., 1996; Field et al., 1995; Partridge et al., 1999b; Zhang et al., 1997, 2000). For example, 3p loss is one of the most common events in both OPLs and oral SCCS, 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; 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, \textit{FHIT} (fragile histidine triad), has been located at 3p14 (Mao et al., 1996c; Mao, 1998; Pateromichelakis \textit{et al.}, 2000; Tanimoto \textit{et al.}, 2000). TSGs at the other deletion regions on 3p may also play important roles in cancer development, although these genes have yet to be identified.
1.3.5. LOH analysis and oral cancer

Recent studies including those from this lab have shown that the loss of specific regions of chromosomes that contain tumor suppressor genes is a common event in oral SCCs (Ah-See et al., 1994; Nawroz et al., 1994). By definition, specific loss of heterozygosity is distinguished from random genetic loss due to generalized chromosomal instability if it occurs in more than 20% of specimens tested for a particular marker (Li et al., 1994). In this thesis, microsatellite markers on chromosome arms 3p, 4q, 8p, 9p, 11q, 13q and 17p were used, since they have been reported to lie within regions most frequently lost in oral SCCs (Table 10) and because of their possible role as risk indicators in each step of the progression of oral cancer (Rosin et al., 2000). Each of these regions will be discussed.

**Chromosome 3.** High frequency of LOH at chromosome 3p has been reported in head and neck cancers (Table 10). The losses appear to center around 3p13-21.1, 3p21.3-23, and 3p24-25 (Maestro et al., 1993; 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 such as esophageal, gastric, colonic, mammary, cervical, small cell lung, and head and neck carcinomas (Mao et al., 1996c; Pennisi, 1996; Sozzi et al., 1996; Wilke et al., 1996; 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*., 1996c).

Until now there is sufficient evidence for only one gene, *FHIT*, as a candidate TSG in the region 3p14.2, although the evidence in support of it being a TSG is still considered to be 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 clear. 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* also mentioned the possibility that the *VHL* gene may be involved in oral SCC development (1998). 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 D3S 1110 at 3p25 (Uzawa *et al*., 1998; Waber *et al*., 1996).

**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% (Bockmihl *et al*., 1996; 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; 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; 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; 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 known 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; Reed et al., 1996).
Mutations of this gene are not apparently frequent for oral cancer (Dawson et al., 1996; Reed et al., 1996). That 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; Rawnsley et al., 1997). Alternatively, another tumor suppressor gene may exist in this region (Dawson et al., 1996; Reed et al., 1996; Waber et al., 1997) which may play a role in aggressive disease as manifest by local, regional, or distant recurrence (Lydiatt et al., 1998; 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; Somers, 1990). Amplification of this region in association with poor prognosis has also been reported (Meredith et al., 1995). 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; 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; Ogawara et al., 1998). There is some data suggesting 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; 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., 1996). 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 cholinergic receptor B1 (CHRNB1) locus at 17p11.1-12, that is tightly linked to the p53 regions (Adamson et al., 1994; 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 genetic 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., 1996). 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., 1999c).
Table 10. LOH frequencies in head and neck or oral cancers

<table>
<thead>
<tr>
<th>Chromosome Arm</th>
<th>Oral Cancer</th>
<th>Head and Neck Cancers</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>17/21 Partridge et al, 1994 (3p21)</td>
<td>8/18 Ah-See et al, 1994</td>
</tr>
<tr>
<td></td>
<td>52-81%</td>
<td>18/27 Califano et al, 1996</td>
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<tr>
<td></td>
<td></td>
<td>18/27 Nawroz et al, 1994</td>
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<tr>
<td></td>
<td></td>
<td>22/46 Rowley et al, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25/50 Gonzalez et al, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44 - 74%</td>
</tr>
<tr>
<td>4q</td>
<td>N/A</td>
<td>20/27 Pershouse et al, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51/100 Shah et al, 2000</td>
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<tr>
<td></td>
<td></td>
<td>51 - 75%</td>
</tr>
<tr>
<td>8p</td>
<td>21/35 Wu et al, 1997</td>
<td>11/36 Li et al, 1994</td>
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<tr>
<td></td>
<td>60-79%</td>
<td>14/40 Field et al, 1995</td>
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<tr>
<td></td>
<td></td>
<td>10/19 El-Naggar et al, 1995</td>
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<tr>
<td></td>
<td></td>
<td>8/20 Califano et al, 1996</td>
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<tr>
<td></td>
<td></td>
<td>13/44 Scholnick et al 1996 (8p264)</td>
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<tr>
<td></td>
<td></td>
<td>23/51 Ishwad et al, 1999 (8p23)</td>
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<tr>
<td></td>
<td></td>
<td>31 - 53%</td>
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<tr>
<td></td>
<td>48%</td>
<td>21/29 Nawroz et al, 1994</td>
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<tr>
<td></td>
<td></td>
<td>24/39 Field et al, 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13/18 El-Naggar et al, 1995</td>
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<tr>
<td></td>
<td></td>
<td>54/74 Califano et al, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17/42 Lydiatt et al, 1997</td>
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<tr>
<td></td>
<td></td>
<td>21/43 Partridge et al, 1999b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 - 73%</td>
</tr>
<tr>
<td></td>
<td>(11q23, 11q25)</td>
<td>9/20 Ah-See et al, 1994</td>
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<tr>
<td></td>
<td>56%</td>
<td>3/39 Field et al, 1995</td>
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<tr>
<td></td>
<td></td>
<td>5/15 el-Naggar et al, 1995 (11q13)</td>
</tr>
<tr>
<td>Chromosomal Arm</td>
<td>Lesions</td>
<td>Study Authors and Year</td>
</tr>
<tr>
<td>-----------------</td>
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<td>------------------------</td>
</tr>
<tr>
<td>13q</td>
<td>23/34</td>
<td>Ogawara et al, 1998</td>
</tr>
<tr>
<td>13q</td>
<td>13/52</td>
<td>Lazar et al, 1998</td>
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<tr>
<td>13q</td>
<td>9/23</td>
<td>Venugoaplan et al, 1998</td>
</tr>
<tr>
<td>12p</td>
<td>N/A</td>
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<tr>
<td>17p</td>
<td>N/A</td>
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</tbody>
</table>

1Data shown as number of lesions showing LOH/total number of informative cases. Numbers in bold face are the range of % LOH for indicated chromosomal arm. N/A, information not available.

### 1.3.6. LOH analysis and oral premalignant lesions

Since tumorigenesis is a sequential accumulation of genetic alterations, analysis of early and late stage lesions may define the genetic changes associated with the development and progression of HNSCC. Few studies (Table 11) have investigated the premalignant stages of the lesions, while there are many studies of LOH on oral SCCS. 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.
The limited number of studies on premalignant lesions 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., 1996d; 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; 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 hyperplasias (Zhang et al., 1997). Several recent studies have shown that FHIT may be significantly involved in oral SCC development (Croce et al., 1999; Mao, 1998) and suggest that alteration to this gene may play an important role in early stage in the development of this cancer (Mao et al., 1996d). It was recently suggested that, in some tissues and organs and particularly those associated with exposure to environmental carcinogens, alterations in FHIT occur quite early during the development of human cancer (Croce et al., 1999). LOH at 3p14 has been shown to be involved in oral premalignant lesions and may also have prognostic significance (Hu et al., 1996; Mao et al., 1996d; Patridge et al., 1996; Rosin et al., 2000; 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). 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). The studies suggested that inactivation of TSG(s) within these loci may constitute an early event in the evolution of oral SCC.

Very few studies on biomarkers have been done on oral verrucous lesions. In the only apparent study on genetics of verrucous lesions (Gopalakrishnan et al., 1997), 10 cases were assayed for \( p53 \) mutation using SSCP (single strand conformation polymorphism) analysis. Although \( p53 \) mutation is common in oral SCC, no \( p53 \) mutation was found in these lesions. To my knowledge, there is no LOH study on verrucous lesions.
Table 11. LOH frequencies in dysplastic lesions

<table>
<thead>
<tr>
<th>Chromosome arm</th>
<th>Oral premalignant lesions</th>
<th>Head and Neck lesions</th>
</tr>
</thead>
</table>
| 3p             | 7/21<sup>1</sup> Roz et al, 1996  
6/32 Mao et al, 1996d  
12/30 Emilion et al, 1996  
8/18 Mao et al, 1998  
27/75 Rosin et al, 2000  
<sup>1</sup>19-44% | 1/19 el-Naggar et al, 1995  
15/29 Califano et al, 1996  
<sup>1</sup>5-52% |
| 4q             | 0/69 Rosin et al, 2000  
<sup>1</sup>14% | NA |
| 8p             | 19/72 Rosin et al, 2000  
<sup>1</sup>26% | 3/24 Califano et al, 1996  
8/30 el-Naggar et al, 1998  
<sup>1</sup>13-27% |
| 9p             | 12/17 van der Riet et al, 1994  
12/32 Mao et al, 1996d  
43/75 Rosin et al, 2000  
<sup>1</sup>38-70% | 17/30 Califano et al, 1996  
6/18 el-Naggar et al, 1995  
17/21 Mao et al, 1998  
<sup>1</sup>33-81% |
| 11q            | 15/75 Rosin et al, 2000  
<sup>1</sup>20% | 9/31 Califano et al, 1996  
1/15 el-Naggar et al, 1995  
<sup>1</sup>7-29% |
| 13q            | 9/74 Rosin et al, 2000  
<sup>1</sup>12% | 9/28 Califano et al, 1996  
<sup>1</sup>32% |
| 17p            | 3/16 Emilion et al, 1996  
22/82 Rosin et al, 2000  
<sup>1</sup>18-27% | 10/30 Califano et al, 1996  
1/14 el-Naggar et al, 1995  
8/19 Mao et al, 1998  
<sup>1</sup>7-42% |

<sup>1</sup>Data shown as number of lesions showing LOH/total number of informative cases.

Numbers in bold face are the range of % LOH for indicated chromosomal arm.

N/A, information not available.
I.3.7. Genetic progression pathway for oral carcinogenesis

The multistep concept of cancer and precancer has developed gradually with increasing evidence that more than one gene is involved. An early indication of this came when incidence of cancer was plotted as a function of age. Using this approach, the necessary number of rate-limiting "hits" can be derived from the slope (Renan, 1993). This data suggested that multiple mutations were required for cancer developed. This multi-gene involvement was put together into a genetic pathway for histo-pathological progression of colon cancer. They suggested that a) tumors progress via the activation of oncogenes and the inactivation of TSGs, each generating a growth advantage for a clonal population of cells; b) specific genetic events often occur in a distinct order of progression; but c) the order of progression is not necessarily the same for each individual tumor, and therefore it is the accumulation of genetic events that determines tumor progression. The multistep genetic process in cancer development is well-established as a model of colorectal carcinoma, in which at least seven genetic events are postulated to be involved. This model links the histological changes that occur in that tissue during the development of cancer to mutation of specific genes. The underlying implication of this model was that for cancer to develop, a critical number of genetic changes had to occur in the tissue and that these changes accumulate during the progression of the tumor and are integral to the development of the pathology. Molecular progression models can define the occurrence and temporal sequence of specific genetic events which take place well before a given tumor produces clinical symptoms and often before a lesion develops into invasive cancer (Sidransky, 1995).

Statistical analysis, based on the age-specific mortality rates for different types of human cancer, indicates that 6-11 critical mutations might be required for the tumorigenesis of head and
neck tumors (Emilion et al., 1996; Renan, 1993). The establishment of a working molecular model in the oral cavity, such as that for the development of colon cancer, would be important not only for our understanding of the mechanisms underlying the early carcinogenesis process in the oral cavity, but also for early diagnosis and management of high-risk oral premalignant lesions before they become invasive.

In a landmark study by Califano and his colleagues (1996), LOH was investigated in 87 premalignant lesions of the upper aerodigestive tract, in a whole spectrum of histologies, including hyperplasia, dysplasia, CIS. The study proposed a genetic progression model for the carcinogenesis of HNSCC (Figure 7). The model proposed 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) together. This may partly be a result of difficulty in obtaining enough samples for each individual degree of dysplasia. Understanding the molecular changes in lesions with different degree of dysplasia is very important as lesions with different degrees of dysplasia would have markedly different behavior (e.g., severe dysplasia versus mild dysplasia) and may represent totally distinct steps in the carcinogenesis process.
To address this problem and refine the molecular model, a plan was made to investigate LOH genetic patterns for each histological category in the oral histological progression model, that is, hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, CIS and SCC, using 19 primers on 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p). As this is an extensive amount of work, various members of the research team participated in this project. Parts of the data generated first have been used in a recent publication with myself as one of the authors (Rosin et al., 2000). This thesis carried out the remaining LOH analyses to complete and refine this model.

1.3.8. LOH analysis and prediction of cancer risk for oral premalignant lesions (OPL)

In a hallmark study, Mao and co-workers (Mao et al., 1996d) investigated oral leukoplakia for the association between LOH at 2 chromosome arms (3p and 9p) and cancer progression. The results showed that the presence of LOH at 9p21 &/or 3p14 in oral leukoplakia was associated with a greater probability of progression of this premalignant lesion into SCC: 7
of 19 (37%) cases with such LOH progressed to SCC in their study, as compared to only 1 of 18 (6%) cases without LOH.

To further investigate the roles of LOH at various chromosomes during early carcinogenesis, another recent study from our lab examined a set of 116 biopsies from patients with OPL (hyperplasia or mild/moderate dysplasia) for LOH at 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p). None of the patients had a history of cancer prior to these OPLs. In 29 of the patients, the lesion later progressed into cancer, whereas the rest did not. The genetic changes of these morphologically similar but behaviorally different lesions were examined and compared in the hope of identifying genetic changes that are characteristic of the progressing high-risk premalignant lesions. The results showed that the two groups of lesions differed in their LOH pattern. Compared to leukoplakia that have not progressed into cancer, progressing lesions showed not only a significantly higher number of LOHs (frequent multiple losses) but also characteristic LOH patterns. Virtually all the progressive lesions (97%) had LOH at 3p and/or 9p, suggesting that loss in these arms is a prerequisite for progression, although such loss alone is probably insufficient for malignant transformation. Additional loss at other chromosome arms significantly increased the cancer risk. Based on these results, 3 risk groups were deduced: a low-risk LOH pattern (retention of 3p and 9p); an intermediate-risk (LOH at 3p and/or 9p) and a high-risk pattern (LOH at 3p and/or 9p plus loss at 4q, 8p, 11q, 13q or 17p). Lesions with the high-risk pattern had a 33-fold increase in cancer risk as compared to those with a low-risk pattern (Mao, 2000; Rosin et al., 2000). This is an important study, since it provided a useful approach for differentiating high-risk lesions from morphologically similar low-grade premalignancies.
One additional study has examined OPLs for an association between LOH patterns and progression. Partridge et al. (2000a) studied biopsies from 78 patients with OPL (hyperplasia or dysplasia) but no prior history of oral cancer. Half of the patients had experienced progression, usually at or adjacent to the originally identified regions of hyperplasia or dysplasia. Loss of 9p or 3p was observed in 94% of lesions that progressed to cancer, significantly higher than those that did not progress. A previous study from Partridge et al. (1998) also showed that multiple LOH predicts cancer progression for OPL. All of the above studies showed LOH might be a promising marker for risk prediction of malignant transformation.

1.3.9. LOH analysis and treatment of OPL

As discussed in Section 1.2.2. (Problems in the management of oral premalignant lesions), there is no agreement as to how OPL, particularly those without dysplasia or with minimal dysplasia, should be managed (e.g. "watch" versus removal). In theory, if we could subgroup the OPL according to malignant risk, the low-risk lesions might be left untreated, but the high-risk lesions removed. As discussed above, the advancement in molecular studies of these lesions has provided a new tool to further categorizing low-grade OPL into different molecular risk groups (Rosin et al., 2000). However, there were no data on whether lesions within different molecular risk groups could be treated differently. This thesis performed a preliminary study to explore the impact of treatment on low-grade oral lesions with different molecular risk profiles.
1.3.10. Oral cancer and $p53$ mutation

Alterations of $p53$ either at the gene or at the protein level are a common feature in many human cancers, including oral cancers. Mutation of the $p53$ gene accompanied by a loss of the other $p53$ allele (located on chromosome 17p13) is the most common genetic alteration in human cancer (Hollstein et al., 1991; Nigro et al., 1989). Several papers have shown that a high percentage of oral SCCs stained positive for the mutated $p53$ products. For example, Kaur et al. (1994) detected positive $p53$ immunostaining in 55% of oral dysplasias and in 75% of oral SCCs. When the stage-wise progression of oral lesions is considered, studies have found that with increasing degrees of dysplasia and the appearance of SCC, not only was there an increase in the percentage of cases demonstrating $p53$ staining, but also an increase in the staining intensity of the positive cells and expansion of these positive cells (Zhang et al., 1993).

Several studies have measured the rate of $p53$ mutation in oral cancer and HNSCC. A high percentage of these tumors contained $p53$ mutation (Ahomadegbe et al., 1995; Boyle et al., 1993; Greenblatt et al., 1994; Saranath et al., 1993; Zariwala et al., 1994). However, abnormal protein expression does not necessarily indicated $p53$ gene mutation (Macgeoch et al., 1993) and the percentage of $p53$ mutation in SCC was lower than that of $p53$ protein over-expression. This variability also reflects the high false-positive and false-negative rates of immunohistochemical staining when applied to formalin-fixed, paraffin-embedded tissue sections (Dowell and Ogden, 1996; Calzolari et al., 1997). The significance of this discrepancy remains unknown and there have been suggestions that overexpression of $p53$ alone without mutation also plays a role in cancer development.
Mutation of the p53 gene and overexpression of the p53 protein are strongly correlated with the use of tobacco products. Lazarus et al., (1996) measured p53 mutation in oral premalignant lesions of non-smokers and smokers. All cases that demonstrated missense mutations in p53 were from tobacco users, whereas non-tobacco users did not exhibit any p53 mutations. In addition, ex-smokers also had a higher incidence of p53 mutations than those who had never smoked.

Few studies, however, have investigated p53 mutations with respect to preinvasive oral lesions, particularly the early dysplastic lesions. In a study by Lazarus et al. (1996), 4 of 26 cases (15%) of oral premalignant lesions demonstrated p53 mutation. In another recent study, p53 mutation was examined in 10 cases of sequential oral epithelial dysplasias and squamous cell carcinoma and the mutation was seen in only 8% (1/12) of dysplasias but 73% (8/11) of carcinomas (Shahnavaz et al., 2000). These results are in agreement with previous studies that showed p53 gene mutation seemed to occur relatively late and is associated with transformation to the invasive phenotype (Boyle et al., 1993; Li et al., 1995).

1.3.11. Relationship between p53 mutation and HPV infection

Oncogenic viruses, such as HPV, have developed several mechanisms for abrogation of p53 function. HPV proteins act, at least in part, by complexing with wild-type p53 protein and degrading it through a proteolytic cell system, known as the ubiquitin pathway (Neil et al., 1997). The proteolytic cell system is able to degrade only wild-type p53. This is true for high-risk HPV-16/-18 types. These high-risk HPVs produce E6 oncoprotein capable of binding and degradation of p53 protein. The outcome is similar to p53 mutation, as normal p53 protein is no
longer functional. Cells with the infection could undergo neoplastic transformation with further DNA damage such as exposure to environmental carcinogens.

Inactivation of the \( p53 \) gene by both mutation and binding to the HPV oncoprotein E6 is not common and might seem unnecessary, but data indicate that both HPV infection and inactivating \( p53 \) could exist simultaneously (Lee et al., 1993; Mao et al., 1996a). The mechanisms behind this remain unclear. The co-existence of HPV and \( p53 \) mutation is an unfortunate event for the host cell, since they act cooperatively to break down its defenses and "seduce" it into the carcinogenic process (Haraf et al., 1996). There are also data indicating that coexistence of HPV and \( p53 \) mutations results in more aggressive, rapidly developing and frequently recurrent oral carcinomas (Mao et al., 1996a; Gopalakreshnan et al., 1997).
CHAPTER II.

TECHNIQUES UTILIZED IN THIS THESIS
II.1. Samples

As in any clinical study of this type, one of the major difficulties in this thesis was case identification. The majority of samples used for this study were from the Provincial Oral Biopsy Service. This centralized Oral Biopsy Service supports dentists and ENT surgeons throughout the province, at no cost to the provider or patient. With more than 3,500 biopsies of oral lesions collected per year (19 years archived), a large number of patients with early lesions can be followed over time. In addition, samples from the Vancouver General Hospital, British Columbia Cancer Agency (BCCA), and several other hospitals were also used.

II.1.1. Databases

Gathering samples for the study involved both manual search (going through the archived pathology report), and search of databases. I have learned to search databases at the British Columbia Cancer Agency and Vancouver General Hospital. Most of the patient files in the Provincial Oral Biopsy Service were either not computerized, or if computerized, the filing systems did not allow easy search. Based on the need, a database has been established for premalignant lesions in the Provincial Oral Biopsy Service by manually searching all premalignant lesions in the archives and entering the information into a database. In order to manage the huge amount of information for the samples used in this study (clinicopathological information, LOH information, p53 mutation information and HPV information), a laboratory ACCESS database was created.
II.1.2. Criteria for choosing samples used included

1) **Histological diagnosis:** A histological diagnosis of a case was confirmed by 2 pathologists using criteria established by the World Health Organization (WHO Collaborating Reference Centre 1978). Dr. Priddy and Dr. Zhang are the two oral pathologists who confirmed the histological diagnoses for the cases used for my thesis.

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

3) **Clinicopathological information:** This was obtained by reviewing all the pathology reports and available clinic or hospital charts. The following data were obtained for the cases studied: age and gender of the patients; date of the biopsy, histological diagnosis, anatomical location of the lesions; and tobacco usage. However, not all of the patients’ charts indicated tobacco habits.

**History of cancer:** All the premalignant lesions used in the study were confirmed to not have a preceding oral cancer. The outcome of these lesions (i.e. whether they later progressed into cancer) was determined. For progressing cases, the time between the date of biopsy for the premalignant lesion and the date of biopsy for the later cancer was recorded. All the SCC used in the study were primary cancer, not recurrent. This information was obtained by using a computer link with the British Columbia Cancer
Registry, where all the cancer in the province must be registered, in addition to review of pathology reports and patient charts as mentioned above.

_Treatment history:_ This information was obtained by contacting the clinicians that managed the OPLs in addition to reviewing pathology reports and patient charts.

II.2. DNA preparation

II.2.1. Slide Preparation

When a case was chosen, the tissue block for the case was removed from the archive, and one slide with 5 micron thick sections was stained with H & E (hematoxylin and eosin) and coverslipped for reference. The actual samples for microdissection were 12 microns thick and each block had approximately 15 sections. They were also stained with H&E but left uncoverslipped.

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

II.2.2. Microdissection

Microdissection of the specimens was either performed or supervised by Dr. L. Zhang, an oral pathologist at UBC. Areas of dysplasia were identified using H&E stained sections cut from formalin-fixed paraffin-embedded tissues. Epithelial cells in these areas were then meticulously dissected from adjacent non-epithelium tissue under an inverted microscope using a 23 G needle. Control DNA was obtained by dissecting cells from the underlying stroma in these sections.

II.2.3. Sample digestion and DNA extraction

The microdissected tissue was placed in a 1.5 ml eppendorf tube and digested in 300 ul of 50 mM Tris-HCL (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and proteinase K (0.5 mg/ml) and incubated at 48°C for 72 or more hours. During incubation, samples were spiked with 10 or 20 ul of fresh proteinase K (20 mg/ml) twice daily. The DNA was then extracted 3 times with PC-9, a phenol-chloroform mixture, 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 submitted for DNA quantitation (Rosin et al., 1997; Zhang et al., 1997).
II.2.4. DNA quantitation

Fluorescence analysis with a Picogreen kit (Molecular Probes) was used to quantitate DNA. This method used 2 standard curves. The low concentration standard curve was used for samples with 1 to 20 ng/ul, while the high concentration standard curve was used for concentrations between 10 and 400 ng/ul. 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 5 ng/ul with LOTE buffer (Rosin et al., 1997; Zhang et al., 1997).

II.3. Microsatellite analysis for LOH

II.3.1. Primer-extension preamplification (PEP)

If the concentration of DNA was low (< 100 ng total DNA), a procedure called primer-extension preamplification (PEP) was done to increase the amount of DNA. This step involved amplification of multiple sites of the genome using random primers and low stringency conditions. It was carried out in a 60 ul 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 uM of random 15-mers (Operon Technologies), and 1 ul of Taq DNA polymerase. 2 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 et al., 1997; Zhang et al., 1997).

II.3.2. End-labeling

One more step prior to PCR was end-labeling of one member of the primer pair. The reaction contained a mixture of PCR standard water, 10× buffer for T4 polynucleotide kinase (New England BioLabs), 10× BSA, one of the primer pair, T4 polynucleotide kinase (New England BioLabs), and [γ-32P] ATP (20 uCi, Amersham). The PCR reaction included 1 cycle at 37°C for 60 min run on the thermal cycler (Rosin et al., 1997; Zhang et al., 1997).

II.3.3. PCR and LOH analysis

All samples were coded in such a way that the analysis of LOH would be performed without knowledge of the sample diagnosis. LOH analysis was done on 3p, 4q, 8p, 9p, 11q, 13q and 17p arms. The microsatellite markers (Research Genetics—Huntsville, AL) used in this analysis 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 (CHRNBI) and 17p13.1 (tp53 and D17S786).
PCR amplification using the thermal cycler was carried out in a 5 ul reaction volume containing 5 ng of genomic DNA, 1 ng of labeled primer, 10 ng of each unlabeled primer, 1.5 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 units of Taq DNA polymerase (Life Technologies), PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH8.8), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide], and 2 drops of mineral oil. Amplification involved 1 cycle of pre-heat at 95°C for 2 minutes; and 40 cycles of 1) denaturation at 95°C for 30 seconds, 2) annealing at 50-60°C (depending on the primer used) for 60 seconds, and 3) polymerization at 70°C for 60 seconds; and 1 cycle of final polymerization at 70°C for 5 minutes. The PCR products were then diluted 1:2 in loading buffer, separated on 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography. The films were then coded and scored for LOH without knowing the diagnosis (Rosin et al., 1997; Zhang et al., 1997).

II.3.4. Scoring of LOH

For informative cases (meaning both alleles were of different length and thus could be distinguished from one another on the gel), allelic loss 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 et al., 1997; Zhang et al., 1997). Theoretically, if a region is lost, it will not be amplified and therefore will not appear on the film. However, since there are always some normal cells included in the cells microdissected from the lesions, and genetic variation exists within the lesion, researchers have adopted a 50% cutoff (Emilion et al., 1996) (i.e. a reduction in the
intensity of one of the alleles by 50% in the lesion DNA compared to normal DNA). This cutoff is arbitrary but nevertheless has been the common standard in LOH studies.

II.4. Molecular detection of HPV infection

II.4.1. End-labeling

Procedures used for sample digestion and DNA extraction were as previously described. The end-labeling procedure used in HPV genome detection is similar to that in LOH assay. To increase the sensitivity of the detection of HPV DNA, both paired primers were end-labeled. The reaction contained a mixture of PCR standard water, 10 x buffer for T4 polynucleotide kinase (New England BioLabs), 10 x BSA, two paired primers (100 ng/µl), T4 polynucleotide kinase (New England BioLabs), and [γ-32P] ATP (20 uCi, Amersham). The PCR reaction included one cycle at 37°C for 60 minutes run on the thermal cycler (for recipe see Table 12). The product can be stored in a refrigerator at 4°C for 3-4 days.
Table 12. Recipe of end-labeling for detection of HPV-DNA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR standard water</td>
<td>34</td>
</tr>
<tr>
<td>10x Polynucleotide Kinase buffer</td>
<td>5</td>
</tr>
<tr>
<td>100x BSA</td>
<td>1.0</td>
</tr>
<tr>
<td>Primer R (100ng/µl)</td>
<td>1.5</td>
</tr>
<tr>
<td>Primer F (100ng/µl)</td>
<td>1.5</td>
</tr>
<tr>
<td>T4 polynucleotide kinase</td>
<td>3.0</td>
</tr>
<tr>
<td>[γ-{superscript}32P] ATP (20 uCi, Amersham)</td>
<td>4.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
</tr>
</tbody>
</table>

II.4.2. PCR for detection of HPV DNA

HPV status was determined by PCR analysis using two degenerative universal primers (MY09/MY11; Manos et al., 1989 and GP5/GP6, Snijders et al., 1990) and two HPV type-specific primers (for primer details see Table 13). Virus type-specific primers were used to identify oncogenic high-risk HPV strains (HPV 16 and 18, Baay et al., 1996). These primers were designed for paraffin-embedded tissue samples. All oligonucleotide primers were obtained from Research Genetics (Huntsville, AL). As an internal control of DNA extraction and PCR, all samples can were amplified using primers for a region on GAPDH gene (225 bp, glyceraldehyde phosphate dehydrogenase gene, a human housekeeping gene, Table 13). The end-labeling procedure for GAPDH primers is the same as that of primers in LOH assay.
PCR amplification was carried out in 5 μl reaction volumes containing 5 ng of genomic DNA; 1 ng of each labeled primer for HPV genome and GAPDH2; 10 ng of each unlabeled primer; 1.5 mM each of dATP, dGTP, dCTP, and dTTP; 0.5 units of Taq DNA polymerase (Life Technologies); and PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM β-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide] (for recipe see Table 14). Amplification involved one cycle of pre-heat at 95°C for 2 minutes; and 30 cycles of 1) denaturation at 95°C for 30 seconds, 2) annealing at 50-60°C (depending on the primer used, see Table 13) for 60 seconds, and 3) extension at 70°C for 60 seconds; and one cycle of final extension at 70°C for 5 minutes. The PCR products were then diluted 1:2 in loading buffer and separated on an 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography.

Negative control included DNA extracted from human blood or archived lymphoid tissue in addition to a tube with reagents only. DNA extracted from HeLa and SiHa cell lines were used as positive control for HPV18 and 16, respectively.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Target</th>
<th>Sequence (5'-3')</th>
<th>Genome position</th>
<th>Amplimer length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY09</td>
<td>L1</td>
<td>CGTCMARRGGAWCTGATC</td>
<td>7014-7033</td>
<td>448-454 (450)</td>
</tr>
<tr>
<td>MY11</td>
<td>L1</td>
<td>GCMCAGGGAATATAAGTGG</td>
<td>6582-6601</td>
<td>(6538-6593)</td>
</tr>
<tr>
<td>GP5</td>
<td>L1</td>
<td>Y1C-TTTTACTGTCTGATAC</td>
<td>6746-6765</td>
<td>95</td>
</tr>
<tr>
<td>GP6</td>
<td>L1</td>
<td>AACTCAATGCAATCAGAGG</td>
<td>6746-6765</td>
<td>115</td>
</tr>
<tr>
<td>TS16</td>
<td>L1</td>
<td>GGTCGGTGGAGCCTGCTAGTCA</td>
<td>491-510</td>
<td>225</td>
</tr>
<tr>
<td>TS18</td>
<td>L1</td>
<td>CCCTGGACGTAAATTITGGG</td>
<td>7119-7100</td>
<td>225</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 14. Recipe of PCR for detection of HPV DNA

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl) per PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR standard water</td>
<td>1.7</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>0.5</td>
</tr>
<tr>
<td>dNTP (25 mM each of dATP, dCTP, dGTP and dTTP)</td>
<td>0.3</td>
</tr>
<tr>
<td>Primer R (100 ng/ul)</td>
<td>0.1</td>
</tr>
<tr>
<td>Primer F (100 ng/ul)</td>
<td>0.1</td>
</tr>
<tr>
<td>Primer GAPDH1 (100 ng/ul)</td>
<td>0.1</td>
</tr>
<tr>
<td>Primer GAPDH2 (100 ng/ul)</td>
<td>0.1</td>
</tr>
<tr>
<td>Taq polymerase (Life, Technologies)</td>
<td>0.1</td>
</tr>
<tr>
<td>Labeled primers for HPV genome</td>
<td>0.5</td>
</tr>
<tr>
<td>Labeled primer for GAPDH2</td>
<td>0.5</td>
</tr>
<tr>
<td>Sample DNA (5 ng/ul)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0</td>
</tr>
</tbody>
</table>

II.4.3. HPV scoring

A positive HPV signal was scored by comparing samples with positive PCR bands with positive controls (1 ng/ul of HeLa DNA and 5 ng of SiHa DNA) for location on gel. The PCR products of these two controls containing HPV genomes were confirmed by direct sequencing. Every sample was done at least twice. For a positive signal, an additional PCR was performed to confirm the result.
II.5. Analysis for p53 mutation

II.5.1. Amplification of p53 genome

Archived template DNA was prepared and quantified as mentioned previously. A 750 bp fragment of the p53 gene encompassing exons 7 and 8 and a 500 bp fragment with exons 5 and 6 were amplified separately. The products of these first PCR were reamplified using nesting primers for each individual exon. The primers used for amplifying exons are summarized in Table 13. Each PCR reaction contained 10% DMSO, 6.7 mM MgCl2, 1.5 mM dNTP, 7 ng/ul of each primer, 0.05 units of Taq polymerase (Life Technologies), and 0.8 ng/ul template DNA in a final volume of 25ul (for recipe see Table 15). Amplification involved one cycle of pre-heat at 95°C for 1 minute; and 40 cycles of 1) denaturation at 95°C for 30 seconds, 2) annealing at 50-60°C (depending on the primer used, see Table 12) for 60 seconds, and 3) extension at 70°C for 1 minute and 30 seconds; and one cycle of final extension at 70°C for 5 minutes. Thermocycling was performed in an Omigene Hybaid Thermocycler.
Table 15. Recipe for amplification of *p53* genome

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl) per flanking PCR</th>
<th>Volume (µl) per nesting PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR standard water</td>
<td>13.4</td>
<td>9.84</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>10% DMSO</td>
<td>1.1</td>
<td>0.66</td>
</tr>
<tr>
<td>dNTP (25 mM each of dATP, dCTP, dGTP and dTTP)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Primer 1 (350 ng/µl)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Primer 2 (350 ng/µl)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Taq polymerase (Life Technologies)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Sample DNA</td>
<td>5.0 (20 ng in total)</td>
<td>10.0 (from first PCR)</td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>
Table 16. Primers used in p53 mutation assay.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Working conc. (ng/μl)</th>
<th>Annealing temp.</th>
<th>Size of the product (bp)</th>
<th>purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX7F</td>
<td>-gagattcatctcaaaa</td>
<td>350</td>
<td>60</td>
<td>750</td>
<td>Flanking primer for both exon 7 &amp; 8</td>
</tr>
<tr>
<td></td>
<td>-gaaatctggcataact</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EX8R</td>
<td>-gtaggaatccagcgtgctgccttccttc</td>
<td>350</td>
<td>60</td>
<td>213</td>
<td>Nesting primer for exon 7</td>
</tr>
<tr>
<td>6SL x7</td>
<td>-gtaggaatccagcgtgctgccttccttc</td>
<td>350</td>
<td>50</td>
<td>220</td>
<td>Nesting primer for exon 8</td>
</tr>
<tr>
<td>7SR1 8AS</td>
<td>-gtaggaatccagcgtgctgccttccttc</td>
<td>350</td>
<td>50</td>
<td>220</td>
<td>Nesting primer for exon 8</td>
</tr>
<tr>
<td>7AS</td>
<td>-cagagccgcatgtgc</td>
<td>30</td>
<td>50</td>
<td>-</td>
<td>Sequence primer for exon 7</td>
</tr>
<tr>
<td>7S</td>
<td>-cttcctgctctcttccttc</td>
<td>120</td>
<td>50</td>
<td>-</td>
<td>Sequence primer for exon 8</td>
</tr>
<tr>
<td>4S 6AL</td>
<td>-gtaggaatctcctgctgccttccttc</td>
<td>350</td>
<td>58</td>
<td>500</td>
<td>Flanking primer for both exon 5 &amp; 6</td>
</tr>
<tr>
<td>5ASS</td>
<td>-aaaccagctccgtgccttc</td>
<td>30</td>
<td>50</td>
<td>-</td>
<td>Sequence primer for exon 5</td>
</tr>
<tr>
<td>X6</td>
<td>-gagacagagcgtgcttc</td>
<td>200</td>
<td>50</td>
<td>-</td>
<td>Sequence primer for exon 6</td>
</tr>
<tr>
<td>5F2 6R</td>
<td>-gagacagacagcgtgcttc</td>
<td>350</td>
<td>58</td>
<td>500</td>
<td>Flanking primer for exon 5 and 6</td>
</tr>
<tr>
<td>5SR1 6AL</td>
<td>-gttaggaatccacgctgccttccttc</td>
<td>350</td>
<td>58</td>
<td>500</td>
<td>Flanking primer for exon 5 and 6</td>
</tr>
<tr>
<td>5S-1</td>
<td>-gattcctcgtgagtccttccttc</td>
<td>350</td>
<td>60</td>
<td>188</td>
<td>Nesting primer for exon 6</td>
</tr>
<tr>
<td></td>
<td>-gattcctcgtgagtgccttccttc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
II.5.2. Purification

PCR products were extracted by PC-9, precipitated using 2M NaClO4 and isopropanol, washed once by 70% ethanol and resuspended into 12 ul distilled water.

II.5.3. Direct sequencing

Amplified DNA was then subjected to cycle sequencing in the presence of [α⁻³²P]ATP-labeled primer using the AmpliCycle sequencing kit (Perkin-Elmer, Branchvurg, NJ) with sequencing primers shown in Table 13, according to the manufacturer’s protocol. The products of the sequencing reactions were then run on a 7% sequencing gel and visualized by autoradiography. All mutations were confirmed on a second sequencing gel. Possible polymorphism of p53 gene could be ruled out by sequencing paired normal connective tissue DNA.
CHAPTER III (PAPER 1).

“A HIGH FREQUENCY OF ALLELIC LOSS IN ORAL VERRUCOUS LESIONS MAY EXPLAIN MALIGNANT RISK”.

Verrucous carcinoma (VC), a variant of SCC, is believed to progress through verrucous hyperplasia (VH) in contrast to the common pathway of dysplasias to SCC. VC was first described by Ackerman in 1948. It has since been reported in various parts of the body (e.g., nasopharynx, larynx, esophagus, skin, bladder, anogenital region), but occurs most commonly in the oral cavity, accounting for 2 to 12% of all oral cancer (Kran and Perezmesa, 1966; Jordan, 1995; Bonnie and Rankin, 1988; Jacobson and Shear, 1972). Compared to SCC, VC has unique features clinically, histologically and prognostically. However, the pathway of VH to VC frequently overlap or cross the pathway of dysplasia to SCC. Not only these verrucous lesions could become dysplastic or further develop into invasive SCC but also they could co-exist at the same time. There is no molecular information available for these verrucous lesions and it remains speculative that the progression of the verrucous lesions is molecularly similar to that of dysplasia to SCC.

The study of the molecular changes of the verrucous lesions also has important clinical implications. VH and VC frequently present as pathological challenges for pathologists and mistaken as reactive hyperplasias because these lesions lack dysplasia (the gold standard for premalignant lesions) and breakthrough of basement membrane (the gold standard in judging cancer). Such diagnostic difficulties are demonstrated by studies showing that diagnosis of VC is frequently established only after multiple biopsies, 2 to 7 in average, especially for small biopsy specimens (Maurizi et al., 1996; Biller et al., 1971). The frequent delay in the diagnosis of VC, a malignant lesion, and VH, a high-risk premalignant lesion (reported malignant transformation rate of VH is over 70%) pose serious problems in the prognosis of these lesions. Other markers, such as molecular ones, are highly desired for the early diagnosis of these lesions.
This study used microsatellite analysis to investigate the molecular changes of VH and VC. As described above, a genetic progression pathway has been established for the histological progression model of dysplasia to SCC based on microsatellite analysis for LOH. A LOH cancer risk model is proposed based on the genetic progression pathway and has been shown to be a powerful predictor of cancer risk of low-grade dysplasias. This study employed similar microsatellite markers on the 7 chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q and 17p) to investigate the LOH patterns of VH and VC, and compared the LOH of these verrucous lesions with reactive epithelial hyperplasia, mild epithelial dysplasia, moderate dysplasia, severe dysplasia/CIS and SCC. The objectives were two-sided. One was to obtain molecular information of VH and VC, and to use these data to build a genetic progression pathway for these verrucous lesions, and to determine whether the molecular progression of VH to VC is similar to that of dysplasia to SCC. The other was to determine whether LOH patterns could be used to differentiate VH/VC from the reactive hyperplastic lesions.

Archival samples were selected from the Provincial Oral Biopsy Service at Vancouver Hospital & Health Sciences Centre. Six groups of lesions were used: epithelial hyperplasia (47 cases), VH (24 cases), VC (17 cases), low-grade dysplasia (54 cases: 31 mild dysplasias and 23 moderate dysplasias), severe dysplasia/CIS (38 cases) and SCC (43 cases). Tissue sections were cut from the paraffin blocks and microdissected. DNA was extracted from the epithelium (lesion) and connective tissue (control), quantitated and analyzed for LOH using 19 microsatellite markers 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 (CHRNBI) and 17p13.1 (tp53 and D17S786).
The data of the verrucous lesions were compared with those of hyperplasia, dysplasia and SCCs. The results showed that VH/VC shared many of the losses present in dysplasia/SCC but differed in two aspects. First, VH/VC showed early acquisition of loss, compared to a gradual accumulation of losses from dysplasias to SCC. For example, multiple loss, an indicator of malignant risk, is not seen in reactive hyperplasia (0% of cases in this study), but increases in frequency with increasing degree of dysplasia (31% of low-grade dysplasia, 76% of high-grade dysplasia) and SCC (80%). In contrast, the loss in VH is frequent (60% with multiple losses) and similar to that of high-grade dysplasia. Second, a strikingly lower frequency of loss at 17p, especially \( tp53 \) locus was noted in VH/VC compared to dysplasia/SCC. Combining this evidence with the morphology of verrucous lesions, human papillomavirus (HPV) may play a role in the pathogenesis of verrucous lesions. Based on these results a genetic progression pathway for these verrucous lesions was proposed.

The finding of high-risk LOH profiles in VH may partly account for the high progression risk seen for VH. The striking differences in LOH pattern between the verrucous lesions and reactive lesions (60% multiple losses in VH vs. 0% in reactive hyperplasia) could be used as a powerful tool in the differential diagnosis of these verrucous lesions from reactive hyperplasia.

In summary, this is the first study to examine the molecular changes in the verrucous lesions. The results show that these verrucous lesions show different but overlap genetic progression pathway compared to the dysplasia and SCCs, and that microsatellite markers could be powerful tool in the early diagnosis of these lesions.
III.2. Objectives

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

2. To use above data to build a genetic progression pathway for VH and VC.

3. To compare the LOH pattern of VH and VC with that of reactive epithelial hyperplasia to determine whether LOH pattern could be used to differentiate VH/VC from the reactive hyperplastic lesions.

4. To compare the genetic progression pathway of VH to VC with that of dysplasia to SCC.

**Note:** The data obtained on allelic loss on the 7 chromosome regions in the cases of hyperplasia, mild epithelial dysplasia, moderate dysplasia, severe dysplasia, CIS and SCC were also used to establish a genetic progression pathway for dysplasia to SCC pathway (yet to be published), and the data contributed to a recent publication (I am an author) from our lab on risk prediction of low-grade oral epithelial dysplasia (Rosin et al., 2000, editorial on the article Mao, 2000). As this paper is focused on verrucous lesions, this genetic pathway was not discussed in the paper, but discussed in Chapter VI (GENERAL DISCUSSION).
III.3. Hypotheses

1. Similar to dysplasia/SCC, VH/VC will be characterized by frequent multiple allelic loss in contrast to a low single allelic loss in reactive hyperplasia.

2. VH/VC will show a genetic pathway similar to that of dysplasia and SCC

III.4. Published paper

III.4.1. Abstract

Verrucous carcinoma (VC), a variant of squamous cell carcinoma (SCC), is distinct from SCC in morphology and behavior. The underlying genetic changes involved in the development of VC and its precursor verrucous hyperplasia (VH) are unknown. This study determined whether chromosomal regions frequently lost during the development of SCC are also lost in the VH/VC variant. Twenty-five VH and 17 VC were analyzed for loss of heterozygosity (LOH) at 19 loci on 7 chromosome arms using microsatellite analysis. These data were compared with those from 47 reactive hyperplasias, 92 dysplasias (54 low- and 38 high-grade) and 41 primary SCCs. The results showed that VH/VC shared many of the losses present in dysplasia/SCC but differed in two aspects. First, VH/VC showed early acquisition of loss, compared to a gradual accumulation of losses from dysplasias to SCC. The LOH pattern of VH was similar to that of high-grade dysplasia and sharply different from reactive hyperplasia. The loss in VH often
involved multiple arms (in 60% of VH vs. 0% of reactive lesions). Only a marginal elevation of loss was observed at 9p \((p = 0.06)\) and 4q \((p = 0.05)\) from VH to VC due to the high degree of loss already present in VH. Second, a strikingly lower frequency of loss at 17p was noted in VH/VC compared to dysplasia/SCC and may indicate human papillomavirus (HPV) involvement. The finding of high-risk LOH profiles in VH may partly account for the high progression risk seen for VH, and also has potentially important clinical implication. The difficult pathological diagnosis of VH/VC from reactive hyperplasia frequently requires repeated biopsies and results in delay in diagnosis and significantly increased mortality/morbidity. Microsatellite analysis might facilitate this differential diagnosis.

### III.4.2. Introduction

Oral squamous cell carcinoma (SCC) is similar to SCC in many other organs (e.g. uterine cervix, skin, larynx and pharynx) in that it is felt to develop in a multi-step fashion through a series of histological stages with increasing risk of developing into invasive cancer, namely: epithelial hyperplasia; mild, moderate, and severe dysplasias; and carcinoma \textit{in situ} (CIS). The presence of dysplasia and the breakage of the basement membrane (i.e. invasion) are the hallmarks for judging malignancy in SCC (Figure 8a). However, verrucous carcinoma (VC, Figure 8b), a variant of the conventional SCC in these organs, has unique clinicopathological features and biological behavior. VC and its precursor lesion verrucous hyperplasia (VH, Figure 8c) are characterized by a prominent verrucous configuration, yet show minimal or no dysplasia and no breakage of the basement membrane. In the oral cavity, VC and SCC differ in site of occurrence, with VC most commonly in cheek and alveolus/gingiva, and SCC on the
ventrolateral tongue and floor of mouth (Jacobson and Shear, 1972). VC does not metastasize and it grows indolently (a 75% 5-year survival rate for VC versus <50% for SCC), although the growth is so relentless that long-term prognosis may still be dismal. Despite these differences, a close relationship also exists between VC and SCC. VH and VC often co-exist with dysplasia and SCC, and VH and VC have been reported to progress into SCC (Shear and Pindborg, 1980; Slootweg and Muller, 1983).

Recent studies using microsatellite analysis have shown the presence of genetic changes detected in the form of loss of heterozygosity (LOH) in oral dysplasia and SCC. The accumulation of changes in hyperplasias and dysplasias is associated with risk of progression (Ah-See et al., 1994; Califano et al., 1996; Field et al., 1995; Mao et al., 1996d; Mao, 2000, ed.; Partridge et al., 1998, Rosin et al., 1997, 2000; Roz et al., 1996; Zhang et al., 1997, 2000), and response of these lesions to treatment (Zhang et al., 2001a). The objective of the current study is to determine whether chromosomal regions frequently lost during the development of conventional SCC are also present in the VH/VC variant. This study used 19 microsatellite loci on 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p) in a set of samples containing VH and VC and, for comparison, reactive hyperplasia, dysplasia and SCC.

III.4.3. Materials and methods

Sample Collection. Specimens were selected from archival paraffin blocks obtained from the Provincial Oral Biopsy Service at Vancouver Hospital & Health Sciences Centre, Vancouver, Canada. Six groups of lesions were used: 1) 47 reactive lesions (14 hyperkeratotic reactive oral lesions that histologically demonstrate hyperkeratosis &/or acanthosis and 33 other
reactive oral lesions including fibroepithelial polyp, mucocele, periodontitis, traumatic ulcer; 2) 25 VH; 3) 17 VC; 4) 54 low-grade dysplasia (mild and moderate); 5) 38 high-grade dysplasia (severe dysplasia and CIS); and 6) 41 invasive oral SCC. The low-grade dysplasia cases were previously analyzed for LOH changes in a separate study (Rosin et al., 2000). Histological diagnoses of the specimens were reconfirmed by two authors (LZ & RP), both oral pathologists. The criteria used for the diagnosis of dysplasia were those established by WHO (1978). Criteria for the diagnosis of VH and VC were those described by Shear and Pindborg (1980).

Tissues Microdissection and DNA Extraction. Areas of epithelial lesions (hyperplasia, VH, VC, dysplasia, CIS, or invasive SCC) were microdissected from sections stained with hematoxylin/eosin. The underlying stroma from each specimen was dissected and used as a source of matched control DNA. The microdissected tissues were digested in 300 μl of 50 mM Tris-HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and proteinase K (0.5mg/ml) at 48°C and spiked twice daily for 72 hours with 20 μl of fresh proteinase K (20 mg/ml). The DNA was extracted with phenol-chloroform and precipitated with ethanol as previously described (Baker et al., 1990). All samples were coded so that LOH analysis was performed without knowledge of diagnosis.

LOH Assay. The microsatellite markers used for LOH analysis came from Research Genetics (Huntsville, AL) and 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 in regions previously shown to be frequently lost in head and neck SCC. The protocol used for LOH analysis was detailed in Zhang et al., 1997. After PCR
amplification, PCR products were separated on 7\% urea-formamide-polyacrylamide gels and visualized by autoradiography. For informative cases, allelic loss was inferred when the signal intensity of one allele was at least 50\% decreased in the DNA sample from a lesion, compared with the corresponding allele in the matching connective tissue DNA. All samples showing allelic loss were subjected to repeat analysis after a second independent amplification.

**Statistical Analysis.** The Fisher's exact, unpaired t-test and one way ANOVA were used for statistical analysis of the results.

### III.4.4. Results

**Demographic analysis of sample sets.** A total of 224 cases were evaluated in this study belonging to 6 study groups (Table 17). These groups did not differ significantly in gender composition. Data on smoking habit (ever smokers) was available for 77\% of cases (172 of 224 cases) with a similar proportion of cases lacking information in the 6 groups. Percent smokers ranged from 49 to 71\%, with no statistical difference between groups. The lowest percentage was observed in the reactive hyperplasia group. There was a trend toward increasing age with histological progression from reactive hyperplasia to low-grade, to high-grade dysplasia and to SCC \( (p < 0.0001) \). No significant difference in age was noted between VH and VC, or between VH/VC and SCC.

**Comparison of allelic loss between VH and reactive hyperplasia.** Reactive lesions are a heterogeneous group. We initially categorized these lesions into 2 subgroups. The first contained lesions that appeared as white patches in the oral cavity and histologically demonstrated hyperkeratosis and/or acanthosis \( (n = 14) \). The second group included
periodontitis, mucoceles, traumatic ulcers, and fibroepithelial polyps (n = 33). However, since these 2 groups did not differ in frequencies of loss at any of the above mentioned arms, we combined these sub-groups together as reactive lesions (Table 18).

LOH frequencies were significantly higher in VH as compared to reactive lesions. The difference was especially striking for multiple losses (> 1 arm lost) which occurred in 15 of 25 (60%) VH (Figure 9a), but in none of the 47 reactive lesions (p = 0.0001). VH consistently showed an elevated loss for each of the chromosome arms examined (p ranges from 0.0001 to 0.04).

**Comparison of VH and dysplasia.** Table 19 compared LOH frequencies of VH with low-grade dysplasias (mild/moderate) and with high-grade dysplasias (severe dysplasia/CIS). In terms of the amount of loss observed, VH more closely resembled a high-grade lesion. VH showed a greater frequency of loss than low-grade dysplasia, apparent as an increase in mean loss (p = 0.0046), any loss (p = 0.081), and multiple loss (p = 0.026). In contrast, there was no significant difference in any of these parameters for VH versus high-grade dysplasias.

When individual arms were considered, VH showed increased loss at 3p, 4q, 11q (p = 0.02 -0.03) and approached significance for LOH at 13q (p = 0.057) when compared to low-grade dysplasias. Similar frequencies for VH and low-grade dysplasias were observed at 9p, 8p and 17p. In contrast, when compared to high-grade dysplasia, VH showed a significantly lower loss only at 17p (P = 0.0007) and marginally for 9p (approaching significance, p = 0.057).

**Comparison of allelic loss between VH, VC and SCC.** Table 20 compares LOH frequencies of VH with VC and VC with SCC (Figure 9b, c). LOH frequencies increased in VC compared to VH, but did not reach statistical significance, reflecting the high degree of LOH already present in VH (any loss, p = 0.07; >1 arm lost, p = 0.081). With respect to specific arms,
9p and 4q showed a marginal increase between VH and VC ($p = 0.062$ and 0.053, respectively). No difference in overall LOH frequencies were found between VC and SCC ($p = 1$). However, the percentage loss at 4q is actually higher in VC than SCC ($p = 0.041$), although this may be due to small sample size. SCC showed significantly increased loss on 17p ($p = 0.037$) compared to VC.

III.4.5. Discussion

Although VC is distinct from SCC in morphology and behavior, the genetic factors that might contribute to these clinicopathological differences are unknown. The development of SCC is associated with specific genetic alterations. Whether these same alterations are involved in the VC phenotype has not yet been demonstrated. The current paper is the first to examine VC, and its precursor VH, for LOH in multiple chromosomal regions frequently altered in dysplasia and SCC.

The data show that VH and VC share many of the losses present in dysplasia and SCC; however, these losses are accumulated differently. Conventional SCC progression is characterized by a gradual accumulation of LOH on different chromosomal arms (Califano et al., 1996; Rosin et al., 2000). For example, multiple loss, an indicator of malignant risk, is not seen in reactive hyperplasia (0% of cases in this study), but increases in frequency with increasing degree of dysplasia (31% of low-grade dysplasia, 76% of high-grade dysplasia) and SCC (80%). Some chromosomal regions are lost early in progression (3p, 9p, and 17p) while others occur later in high-grade dysplasias or SCC (4q, 8p, 11q, 13q) (Rosin et al., 2000). In contrast, VH, as a precursor lesion, has a strikingly different LOH profile from that of reactive hyperplasia or
low-grade dysplasia. The loss in VH is frequent (60% with multiple losses) and often involves chromosome regions that are typically present only in high-grade dysplasia or SCC. Indeed, VH and high-grade dysplasia differ only at one arm, 17p (significantly lower in VH). The transition from VH to VC involves increases at 9p and 4q (approaching statistical significance, Table 20). VC is similar to SCC in LOH patterns except for lower frequencies of loss at 17p again.

The mechanism(s) that underlie such rapid accumulation of losses and the strikingly lower frequency of LOH at 17p remain speculative. The possibility of genetic instability in these lesions needs to be considered. Another possibility is a difference in etiology. The presentation of a remarkably verrucous configuration and cytological features of viral infection in VC have led to the conjecture of a viral etiology (Eisenberg et al., 1985). This supposition has only been supported by inconsistent reports of human papillomavirus (HPV) DNA in these verrucous lesions, with detection frequencies varying from 0-87.5% of cases (Shroyer et al., 1991, 1993; Palefsky et al., 1995). In a recent comprehensive study, Sidransky and co-workers reported a low rate of HPV in oral SCC (12% of cases) (Gillison et al., 2000). However, other studies have reported different frequencies (Ostwald et al., 1994; Snijders et al., 1996). HPV etiology for oral cancer may be better evaluated with improvement in detecting different types of HPV. Our data, that VC and VH show a strikingly lower frequency of loss at 17p (near or in the tp53 locus) in VC compared to SCC, is consistent with the hypothesis of an HPV etiology for the verrucous lesions. The low rate of 17p loss could be explained by the assumption that p53 protein is inactivated by HPV protein through a ubiquitous pathway instead of through p53 mutation and chromosome losses (Gopalakrishnan et al., 1997).

It is well documented that VH is a high-risk premalignant lesion (with a reported malignant transformation rate of 70%, Silverman and Gorsky, 1997). In fact, some even regard
VH as an early or variant form of VC instead of a premalignant lesion (Murrah and Batsakis, 1994; Slootweg et al., 1983). This high probability of transition may, in part, be explained by the LOH profiles in VH. Not only are these profiles similar to those seen in high-grade dysplasia (with the exception of lower 17p, Table 19) but also they resemble profiles characteristic of low-grade dysplasia at risk of progressing into cancer (Rosin et al., 2000).

The finding of the high-risk LOH profiles in the verrucous lesions has important clinical implications. The lack of dysplasia or breakage of basement membrane makes it difficult to differentiate VH from reactive hyperplasia. Without these gold standards, diagnosis of these lesions is often challenging for pathologists. In fact, some studies have shown that it took an average of 3-4 biopsies before a correct diagnosis of VH or VC could be made (Biller et al., 1971; Jordan, 1995). The sharp differences in LOH patterns between reactive hyperplasia and VH/VC suggest that microsatellite analysis may be a powerful tool to help pathologists in the difficult differential diagnosis of verrucous lesions from reactive lesions, hence improving prognosis of both VH and VC, since delay in diagnoses may result in anaplastic transformation of these lesions to SCC with increased mortality and morbidity (Hansen et al., 1985; Shear and Pindborg, 1980; Silverman et al., 1997).
Table 17. Demographic information

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th># of cases</th>
<th>% male</th>
<th>Mean age (95% CI)</th>
<th>% smokers (ever)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive oral lesions</td>
<td>47</td>
<td>60%</td>
<td>47 (42-51)</td>
<td>49%</td>
</tr>
<tr>
<td>Low-grade dysplasia</td>
<td>54</td>
<td>44%</td>
<td>54 (50-58)</td>
<td>59%</td>
</tr>
<tr>
<td>High-grade dysplasia</td>
<td>38</td>
<td>55%</td>
<td>62 (58-66)</td>
<td>57%</td>
</tr>
<tr>
<td>VH</td>
<td>25</td>
<td>44%</td>
<td>62 (56-68)</td>
<td>68%</td>
</tr>
<tr>
<td>VC</td>
<td>17</td>
<td>47%</td>
<td>69 (63-75)</td>
<td>71%</td>
</tr>
<tr>
<td>SCC</td>
<td>43</td>
<td>59%</td>
<td>63 (60-67)</td>
<td>66%</td>
</tr>
</tbody>
</table>

Table 18. VH vs. reactive oral lesions

<table>
<thead>
<tr>
<th></th>
<th>Reactive lesions</th>
<th>VH</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>47</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Mean loss</td>
<td>0.19 ± 0.4</td>
<td>2.28 ± 1.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Any loss</td>
<td>9/47 (19%)</td>
<td>20/25 (80%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;1 arm lost</td>
<td>0/47 (0%)</td>
<td>15/25 (60%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>LOH on: 3p</td>
<td>5/44 (11%)</td>
<td>13/25 (52%)</td>
<td>0.0004</td>
</tr>
<tr>
<td>9p</td>
<td>1/46 (2%)</td>
<td>11/24 (46%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>4q</td>
<td>0/43 (0%)</td>
<td>7/23 (30%)</td>
<td>0.0003</td>
</tr>
<tr>
<td>8p</td>
<td>0/45 (0%)</td>
<td>7/24 (29%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>11q</td>
<td>2/44 (5%)</td>
<td>8/24 (33%)</td>
<td>0.003</td>
</tr>
<tr>
<td>13q</td>
<td>1/43 (2%)</td>
<td>4/22 (18%)</td>
<td>0.04</td>
</tr>
<tr>
<td>17p</td>
<td>0/45 (0%)</td>
<td>5/25 (20%)</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Table 19. VH vs. dysplasia

<table>
<thead>
<tr>
<th></th>
<th>VH</th>
<th>Low-grade dysplasia&lt;sup&gt;1&lt;/sup&gt;</th>
<th>&lt;sup&gt;p&lt;/sup&gt; value&lt;sup&gt;2&lt;/sup&gt;</th>
<th>High-grade dysplasia</th>
<th>&lt;sup&gt;p&lt;/sup&gt; value&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>25</td>
<td>54</td>
<td>-</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>Mean loss</td>
<td>2.28 ± 1.7</td>
<td>1.19 ± 1.48</td>
<td>0.0046&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.82 ± 1.61</td>
<td>0.21</td>
</tr>
<tr>
<td>Any loss</td>
<td>20/25 (80%)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>32/54 (59%)</td>
<td>0.081</td>
<td>36/38 (95%)</td>
<td>0.10</td>
</tr>
<tr>
<td>&gt;1 arm lost</td>
<td>15/25 (60%)</td>
<td>17/54 (31%)</td>
<td>0.026</td>
<td>29/38 (76%)</td>
<td>0.26</td>
</tr>
<tr>
<td>LOH on: 3p</td>
<td>13/25 (52%)</td>
<td>13/53 (25%)</td>
<td>0.022</td>
<td>21/38 (55%)</td>
<td>1</td>
</tr>
<tr>
<td>9p</td>
<td>11/24 (46%)</td>
<td>24/52 (46%)</td>
<td>1</td>
<td>27/37 (73%)</td>
<td>0.057</td>
</tr>
<tr>
<td>4q</td>
<td>7/23 (30%)</td>
<td>4/48 (8%)</td>
<td>0.031</td>
<td>13/33 (39%)</td>
<td>0.58</td>
</tr>
<tr>
<td>8p</td>
<td>7/24 (29%)</td>
<td>8/51 (15%)</td>
<td>0.22</td>
<td>9/32 (28%)</td>
<td>1.0</td>
</tr>
<tr>
<td>11q</td>
<td>8/24 (33%)</td>
<td>6/52 (12%)</td>
<td>0.030</td>
<td>6/37 (16%)</td>
<td>0.13</td>
</tr>
<tr>
<td>13q</td>
<td>4/22 (18%)</td>
<td>2/53 (4%)</td>
<td>0.057</td>
<td>3/33 (9%)</td>
<td>0.419</td>
</tr>
<tr>
<td>17p</td>
<td>5/25 (20%)</td>
<td>11/54 (20%)</td>
<td>1</td>
<td>24/37 (65%)</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

<sup>1</sup>Cases reported in Rosin et al., 2000.

<sup>2,3</sup>Comparison of VH against "Low-grade dysplasia", and "High-grade dysplasia", respectively.

<sup>4</sup>Values given as number of samples showing loss/total number of informative cases (% of cases in parentheses)
### Table 20. VC vs. VH and SCC

<table>
<thead>
<tr>
<th></th>
<th>VC</th>
<th>VH</th>
<th>p value&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SCC</th>
<th>p value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cases</td>
<td>17</td>
<td>25</td>
<td>-</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>Mean loss</td>
<td>3 ± 1.32</td>
<td>2.28 ± 1.7</td>
<td>0.15</td>
<td>3.6 ± 1.8</td>
<td>0.22</td>
</tr>
<tr>
<td>Any loss</td>
<td>17/17 (100%)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>20/25 (80%)</td>
<td>0.070</td>
<td>39/41 (95%)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;1 arm lost</td>
<td>15/17 (88%)</td>
<td>15/25 (60%)</td>
<td>0.081</td>
<td>33/41 (80%)</td>
<td>0.71</td>
</tr>
<tr>
<td>LOH on: 3p</td>
<td>10/17 (59%)</td>
<td>13/25 (52%)</td>
<td>0.76</td>
<td>29/40 (73%)</td>
<td>0.36</td>
</tr>
<tr>
<td>9p</td>
<td>13/17 (77%)</td>
<td>11/24 (46%)</td>
<td>0.062</td>
<td>27/39 (69%)</td>
<td>0.75</td>
</tr>
<tr>
<td>4q</td>
<td>11/17 (65%)</td>
<td>7/23 (30%)</td>
<td>0.053</td>
<td>13/39 (33%)</td>
<td>0.041</td>
</tr>
<tr>
<td>8p</td>
<td>6/17 (35%)</td>
<td>7/24 (29%)</td>
<td>0.74</td>
<td>20/39 (51%)</td>
<td>0.38</td>
</tr>
<tr>
<td>11q</td>
<td>5/16 (31%)</td>
<td>8/24 (33%)</td>
<td>1</td>
<td>20/40 (50%)</td>
<td>0.24</td>
</tr>
<tr>
<td>13q</td>
<td>2/15 (13%)</td>
<td>4/22 (18%)</td>
<td>1</td>
<td>15/38 (39%)</td>
<td>0.10</td>
</tr>
<tr>
<td>17p</td>
<td>4/16 (25%)</td>
<td>5/25 (20%)</td>
<td>0.72</td>
<td>24/40 (60%)</td>
<td>0.037</td>
</tr>
</tbody>
</table>

<sup>1</sup>VH versus VC,

<sup>2</sup>VC versus SCC,

<sup>3</sup>Values given as number of samples showing loss/total number of informative cases (% of cases in parentheses)
Figure 8. a, Photomicrograph of an invasive SCC. Solid arrows illustrate areas of tumor invasion with breakage of the basement membrane. Hollow arrow shows islands of invasive tumor cells deep in the connective tissue. b, Photomicrograph of a VC. Solid arrow shows that tumor growth is mainly endophytic with large bulbous rete ridges pushing downward relative to the normal location of the overlying oral epithelium (hollow arrow). The basement membrane is still intact. c, Photomicrograph of a VH. The verrucous growth is exophytic (solid arrow indicates the normal location of the oral epithelium), and the basement membrane is intact.
Figure 9. Microsatellite analysis for 3 selected cases.

Informative microsatellite markers were amplified from DNA derived from areas of epithelium containing lesion (including VH, VC and SCC) or normal connective tissue stroma (C). Microsatellite markers being assayed are indicated above each block.

a, Multiple LOH in a case of VH (loss of the lower allele at D3S1300 and the upper allele at D9S171 and INT2).

b, Multiple LOH in a case of VC (loss of the upper allele at D3S1228 and the lower allele at D9S171 and FABP).

c, Multiple LOH in a case of SCC (loss of the upper allele at D3S1228 and tp53 and loss of the lower allele at D8S261 and D11S1778).
CHAPTER IV (PAPER 2).

"IMPACT OF LOCALIZED TREATMENT IN REDUCING RISK OF PROGRESSION OF LOW-GRADE ORAL DYSPLASIA: MOLECULAR EVIDENCE OF INCOMPLETE RESECTION".

IV.1. Thesis summary for manuscript

Currently, there is no consensus on the appropriate treatment for oral premalignant lesions (OPL), particularly those with no dysplasia or with low-grade dysplasia. This is mainly due to the difficulty in predicting outcome for this heterogeneous group of lesions, and due to the fact that only a small portion of OPLs with no or minimal dysplasia progress into cancer. Consequently clinicians disagree on whether these lesions should be treated, or left observed. Even those who choose to treat disagree on how to treat. When a lesion is removed, a large percentage of clinicians simply remove the clinically visible lesion without margin, while others would remove these lesions with a small, normal-looking margin (e.g., 2-4 mm). Only rarely do surgeons remove an oral premalignant lesion with a wide margin. Ten to thirty-five percent of recurrence rates indicate that such removal may not be sufficient (Lummerman et al., 1995; Chiesa, 1993).

To further categorize the malignant risk of the morphologically low-risk OPLs for improvement in their management, a recent study from this lab have shown that LOH patterns may differentiate oral premalignant lesions into 3 progression risk groups: low, with retention of 3p and 9p; intermediate, with losses at 3p and/or 9p; and high, with loss at 3p and/or 9p plus loss at 4q, 8p, 11q, 13q, or 17p (Rosin et al., 2000; editorial on the article by Mao, 2000). The value of this data lies in its potential use to triage patients into different management regimens, that is, the morphologically similar lesions, such as low-grade dysplasias, could be managed differently according to their molecular risk pattern. It could be hypothesized that those lesions with low-grade dysplasia and low-risk genotype (LOH pattern) could be left observed as they rarely progress into cancer; whereas morphologically similar lesions with intermediate- or high-risk LOH patterns need to be completely removed to avoid their progression.
This study was designed to test this hypothesis with two objectives. One was to determine whether treatment had an impact on progression risk of oral premalignant lesions, and if so, whether lesions with different LOH risk profiles responded differently to treatment. Another was to assess whether completeness of lesion removal was a major factor in recurrence.

Sixty-six mild and moderate dysplasias were obtained from the archive of the Provincial Oral Biopsy Service of the British Columbia. The outcome of these dysplasias, i.e., whether later progressed into cancer, was confirmed by accessing the British Columbia Cancer Registry, which tracks all histologically confirmed cases of cancer diagnosed in the province. A detailed clinical information, particularly treatment information, was constructed by careful review of patients’ chart and pathology report as well as by questionnaire to the attending clinicians. The review allowed us to identify patients with repeated biopsies.

Information on the LOH at 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p), and hence the LOH risk pattern for the majority of the 66 cases were already known from the previous study (Rosin et al., 2000). The samples with no information on the LOH patterns and the repeated biopsies identified above were microdissected, and analyzed for LOH at the above 7 chromosome arms using the same 19 primers as the previous study, that is, 3p14.2 (D3S1234, D3S1228, D3S1300); 4q26 (FABP2); 4q31.1 (D4S243); 8p21.3 (D8S261); 8p23.3 (D8S262, D8S264); 9p21 (IFNA, D9S171, D9S736, D9S1748, D9S1751); 11q13.3 (INT2); 11q22.3 (D11S1778); 13q12.3-13 (D13S170); 13q14.3 (D13S133); 17p11.2 (CHRNB1) and 17p13.1 (tp53 and D17S786). The LOH patterns of the follow-up repeat biopsies were compared with those of the primary (index) lesion to see whether both contained the same losses (allelic specific mutations), which indicate incomplete removal.
The results showed that there was no agreement on whether to treat or not treat the low-grade lesions. While 62% of the cases were treated by clinical removal but with varying width of margins, more than one third were not treated and the decision to treat was not influenced by known risk factors (degree of dysplasia, site and clinical appearance of leukoplakia). This again confirms the need for new criteria in the guiding of the treatment of these lesions.

Although treatment guided by clinical removal of leukoplakia reduced cancer progression risk in all 3 risk groups, the amount of reduction in our study group did not reach statistical significance. However, complete removal judged clinically was shown to be frequently inadequate since 8 of 17 cases judged clinically removed contained the same molecular clones in the initial and subsequent biopsies, suggesting incomplete removal. Consequently, when molecular information was included in the assessment of lesion removal, treatment significantly reduced the risk of progression for cases with intermediate \( (p = 0.043) \) and high risk \( (p = 0.001) \) genetic profiles, but not cases with low-risk profiles. Of 17 cases with low-risk LOH profile that were not completely removed, only 1 progressed into cancer. In contrast, removal judged morphologically and molecularly generated a 9.1 fold decrease in progression risk for those with high-risk profile. Altogether, these data suggest the use of molecular profiles to guide the treatment of low-grade dysplasia. Our data also suggest that currently an inadequate margin may in part be responsible for the high rate of recurrence, especially in high-risk lesions.

In summary, the data collected in this study strongly support that molecular markers could be used to triage patients and provide the clinician with an improved ability to target intervention specifically to high-risk lesions. Lesions with low-risk morphology and LOH pattern (the bulk of OPLs) might be left untreated and perhaps only monitored occasionally for clinical or molecular change, thus sparing a large number of patients invasive surgical
procedures. In contrast morphologically similar low-grade lesions with intermediate- and high-risk LOH pattern need to be treated. Those with intermediate-risk pattern might be treated conservatively or be given a chemopreventive regime; whereas those with high-risk LOH pattern might require more aggressive treatment, either with a chemotherapeutic drug, or if surgery were used, with wide excision margins.

IV.2. Objectives

To determine how low-grade dysplasia with different risk molecular profiles respond to treatment.

IV.3. Hypothesis

1. Treatment has no effect on the cancer progression rate of leukoplakia with low-grade dysplasia and low-risk genotype (LOH pattern) as they rarely progress into cancer.

2. Treatment should significantly reduce cancer risk of leukoplakia with high-risk genotype (LOH pattern). Those lesions that continue to progress regardless of removal may have been inadequately removed.
IV.4. Published paper

IV.4.1. Abstract

Currently, there is no consensus on the appropriate treatment for low-grade oral dysplasia. This is mainly due to the difficulty in predicting outcome for this heterogeneous group of lesions. In this study, we constructed a detailed clinical history of 66 mild and moderate dysplasias in order to determine how treatment affected outcome, and to evaluate the effect of treatment on lesions with different genetic profiles, which are defined by patterns of loss of heterozygosity (LOH) associated with low, intermediate and high risk of progression (Rosin et al., 2000). The results showed that although treatment guided by clinical removal of leukoplakia reduced cancer progression risk in all 3 risk groups, the amount of reduction in our study group did not reach statistical significance. To assess whether completeness of lesion removal was a major factor in recurrence, repeat biopsies at the primary sites were analyzed for persistent LOH status on chromosomes 3p, 4q, 8p, 9p, 11q, 13q and 17p. Strikingly, 8 of 17 cases judged clinically removed contained the same molecular clones in the initial and subsequent biopsies, suggesting incomplete removal. When molecular information was included in the assessment of lesion removal, treatment significantly reduced the risk of progression for cases with intermediate \( p = 0.043 \) and high risk \( p = 0.001 \) genetic profiles, but not cases with low-risk profiles. A 9.1 fold decrease in progression risk was observed for those with high-risk profile. Altogether, these data suggest the use of molecular profiles to guide the treatment of low-grade dysplasia. Our data also suggest that currently an inadequate margin may in part be responsible for the high rate of recurrence, especially in high-risk lesions.
IV.4.2. Introduction

There is a continuing debate on how oral premalignant lesions should be managed (Allen, 1998; McCartan, 1998; Scully, 1995; Tradati et al., 1997). These lesions, which typically appear as leukoplakia, are a heterogeneous group that vary in potential for malignant transformation with only a fraction eventually progressing into cancer (Bosatra et al., 1997; Lee et al., 2000; Silverman et al., 1984). Some argue that removal confers only a small risk reduction and hence, recommend that leukoplakia should just be clinically monitored. Others favor a more aggressive approach. In theory, the majority of non-progressing leukoplakia could be left untreated, restricting aggressive therapy to those with high risk for malignancy. However, at present, our ability to identify high-risk leukoplakia is limited.

Currently, histological criteria (the presence and degree of dysplasia) represent the gold standard for judging malignant risk of leukoplakia (Bosatra et al., 1997; Lovas, 1989; Scully, 1995; Silverman et al., 1984). High-grade dysplasia are readily identifiable, and are generally treated aggressively (e.g., removed with a normal looking margin). However, there is no consensus on treatment regimes for leukoplakia without dysplasia or with low-grade dysplasia. Consequently, many of these lesions are left untreated or removed with little or no margin. A high-rate of recurrence (10-35%) indicates that such removal may not be sufficient (Lumerman et al., 1995; Chiesa, 1993). Unfortunately, these lesions represent the bulk of leukoplakia and account for the majority of cases that later progress to cancer (Silverman et al., 1984).

Recent studies support the use of molecular tools as an adjunct to histology to further categorize the cancer risk of those leukoplakia without dysplasia or with minimal dysplasia.
(Califano et al., 1996; Partridge et al., 2000a; Zhang et al., 1997, 2000). In a pioneer study, Mao and co-workers examined oral leukoplakia collected during a chemoprevention trial for chromosomal loss at 9p21 and 3p14 and demonstrated that the loss of these regions was correlated with a greater probability of progression to cancer (Mao et al., 1996d).

In a recent study, we evaluated the use of allelic loss to predict malignant risk for oral hyperplasia and low-grade oral epithelial dysplasia. We compared LOH frequencies in cases known to progress to CIS/SCC with those with no history of progression using 19 microsatellite loci on 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p). We deduced 3 risk groups based on genetic profile: a low-risk LOH pattern (retention of 3p and 9p); an intermediate-risk (loss at 3p and/or 9p) and a high-risk pattern (loss at 3p and/or 9p plus loss at 4q, 8p, 11q, 13q or 17p). Leukoplakia with loss of heterozygosity (LOH) at sites on 3p &/or 9p had a 3.8-fold increase in relative risk of developing cancer. Lesions with additional loss on other chromosome arms had a 33-fold increase in cancer risk as compared to those which retained 3p and 9p (Rosin et al., 2000; Mao, ed., 2000). The impact of treatment regime on outcome of lesions with different molecular profiles was not examined.

In this study we reconstructed a detailed clinical history of 66 low-grade dysplastic lesions by chart review and by questionnaire. The objective was to determine whether treatment had an impact on progression risk, and, if so, whether biopsies with different molecular profiles responded differently to treatment.

IV.4.3. Materials and methods
Sample collection. Cases were selected from the Provincial Oral Biopsy Service of British Columbia on the basis of having a histological diagnosis of mild or moderate dysplasia (confirmed by 2 pathologists, LZ and RP using criteria established by the World Health Organization, Pindborg et al., 1997) and a specimen size that would provide sufficient DNA for multiple LOH analyses. This biopsy service has existed since 1979 and is used by dentists and otolaryngologists throughout the province, archiving approximately 3,800 cases/year. In a previous study we identified a substantial cohort of cases with mild and moderate dysplasia with known outcome (Rosin et al., 2000). However, not all of these cases had complete treatment history. Sixty one of the 77 cases from that study were employed in the current study. Five additional cases with complete clinical history were identified and added to this study group. The current study group of 66 cases consists of 46 cases that did not progress to cancer (called non-progressing cases) and 20 cases that had a history of progression to CIS or SCC (called progressing cases). Forty-two of the 46 non-progressing cases (91%) and 19 (95%) of the progressing cases were present in the previous publication.

Clinical and treatment information. The progression of lesions to SCC was confirmed by accessing the British Columbia Cancer Registry, which tracks all histologically confirmed cases of cancer diagnosed in the province. Treatment and clinical information was obtained by chart review and by questionnaires to primary care dentists or otolaryngologists. We collected information on lesion site, size and clinical appearance (homogeneous vs. non-homogeneous), whether or not the lesion was removed, and, if so, extent of removal as judged by clinical examination, and the technique of removal. However, information on the width of normal margin included in the removal (extent of removal) was mostly not recorded in the charts. Histological assessment was not used as an indicator of extent of removal of lesions because
many of the lesions were removed by laser ablation after diagnostic biopsy. For cases with clinical removal, a history of recurrence and/or progression was collected. Although information was requested on tobacco usage, this information was incomplete (Table 21).

All pathology reports were reviewed for each clinical lesion. This allowed us to identify patients with repeated biopsies. These new specimens were collected and analyzed in order to determine whether they represent clonal evolution from the index (first) biopsy and, from the same data, to obtain molecular evidence for incomplete excision.

**Molecular analysis.** Procedures used for microdissection, DNA extraction and LOH analysis are as published (Zhang *et al.*, 1997). The microsatellite markers used for LOH analysis came from Research Genetics (Huntsville, AL) and 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, D9S736, D9S1748, D9S1751*); 11q13.3 (*INT2*); 11q22.3 (*D11S1778*); 13q12.3-13 (*D13S170*); 13q14.3 (*D13S133*); 17p11.2 (*CHRNA1*) and 17p13.1 (*tp53* and *D17S786*). These markers are localized to regions previously shown to be frequently lost in head and neck tumors.

For the progression cases, we evaluated clonal evolution in recurring lesions and in the final cancer biopsy by LOH analysis at the aforementioned loci. The LOH pattern of the primary progressing dysplasia was compared to that of the corresponding recurrent lesions and cancer.

**Statistical analysis.** Clinicopathological features of patients in progressing and non-progressing groups were compared using either Fisher’s exact test or, for age and follow-up time, unpaired t-test (Table 21). Fisher's exact test was also used to compare histological, clinical and molecular features in lesions that were removed and not removed. Time-to-progression curves were estimated by the Kaplan-Meier method and comparisons were performed using log-rank
test. Relative risks were determined using Cox regression analysis. A $p$ value of 0.05 or less was considered significant and all tests were 2-sided.

IV.4.4. Results

**Demographics of Study Population.** A total of 66 patients with mild and moderate dysplasia were evaluated and their characteristics are shown in Table 21. Patient ages ranged from 24 to 82 years with an average of 56. Thirty-six (55%) of the cases were male. An equal number of mild and moderate dysplasias were present. Forty-two of the dysplasias were located at sites deemed to be locations with an elevated risk of progression (Mashberg and Meyer, 1976; Moore and Catlin, 1967): the floor of the mouth ($n = 15$), ventrolateral tongue ($n = 18$) and soft palate complex ($n = 9$), with the remaining 24 cases located at low-risk sites (the rest of the oral cavity). Thirty-one of the cases had data on clinical appearance of which the majority (77%) were non-homogeneous, a feature associated with elevated risk of progression (Schepman and van der Waal, 1995). With respect to tobacco habits, data were available for 58 cases (9 non-smokers and 49 smokers).

The lesions were classified as progressing ($n = 20$) or non-progressing ($n = 46$), based on whether or not they later developed into SCC. Progressing and non-progressing subgroups did not differ significantly for any of the aforementioned parameters (Table 21). The non-progressing group was followed for a mean of 90 months and the progressing group for 48.5 months.

**Treatment for premalignant lesions.** We divided the cases into 2 groups. The first group (25 of 66 cases, 38%) had an incisional biopsy with only a sample taken for diagnoses
without removal of the lesion. These cases were categorized as "not treated" (Table 22). Of the remaining 41 cases, 35 had the entire clinical lesion removed by surgery or laser and 6 received treatment with topical bleomycin, with disappearance of the clinical lesion after treatment. These cases were categorized as "treated".

During the process of evaluating the treatment of these premalignant lesions, we looked for an association of decision to treat with presence of known clinical risk indicators (e.g., degree of dysplasia, site and appearance of lesion). None of these risk factors showed an association (Table 22). A similar proportion of mild and moderate dysplasias received treatment, as did lesions at high-versus low risk sites or with homogeneous versus non-homogeneous appearance.

**Effect of treatment judged by clinical removal on progression of dysplasias with different genetic patterns.** The study cases were categorized into 3-risk groups based on their LOH pattern (Rosin *et al.*, 2000) and then divided into "treated" and "not treated" groups. The effect of treatment on disease progression was examined using the Fisher's exact test (Table 23) and with the Kaplan-Meier method. Time-to-progression curves were plotted as a function of removal for the 3 risk groups. As shown in Fig. 3, when treatment was judged by clinical removal, there was a trend of lower risk of progression for all 3 risk groups (Figure 12a, b, c). However, the reduction was not significant in any of the 3 risk-groups (p value varied from 0.115 to 0.262 for Kaplan-Meier).

**Can molecular criteria improve treatment assessment and hence outcome?** A possible explanation for the lack of statistical significance is that sometimes clinical assessment of the completeness of treatment is inadequate. This possibility is supported by our data: despite treatment, 10 of 20 leukoplakias with a high-risk LOH pattern progressed into cancer. An actual proof of inadequate treatment would require examination of margins surrounding the clinical
lesion for dysplastic cells or for the same molecular profile as the lesion. Unfortunately, margins are not generally collected from dysplasia. However, we were able to collect repeat biopsies from 19 patients in this study for clonality studies. We examined the LOH patterns (18 loci on 7 chromosomes) in 51 biopsies to assess whether a recurring lesion at the same site represented a re-growth of residual cells (Figure 10). We assumed that such an outgrowth would have a near identical genetic profile as the initial genetic clone, with the possible addition of new changes (example of clonal evolution shown in Figure 11). However, we recognized that, in some cases, the progenitor population may not have been homogeneous. In such instance, the outgrowth of a less dominant subclone would lead to an allelotype that had most but not all alterations, yet still represent the recurrence of the initial population. For this reason, we established a stringent criterion for the molecular evidence of recurrence, tolerating only 1 inconsistency in the allelotype that couldn't be attributed to the outgrowth the initial dominant clone, for example, the loss of a different allele in subsequent biopsies or the retention of a region that had shown loss in the initial biopsies.

Of the 19 cases we examined, 17 showed clonality by the criteria described above, supporting incomplete removal of the initial lesion. In several cases repeated attempts were made to eradicate the lesion, yet clones present in the initial lesion were found in the SCC (Figure 10). Strikingly, 8 of the 17 cases showing clonality had been incorrectly judged by clinical criteria to have been successfully treated.

When the treatment was assessed by a combination of molecular and clinical criteria (addition of molecular evidence for residual clones to clinical information on extent of removal of leukoplakia or recurrence of lesions), we observed a significant reduction in the risk of progression for cases with intermediate- \( p = 0.043, \) Figure 12e and high-risk \( p = 0.001, \) Figure
12f) genetic profiles (9.1 fold decrease in progression risk for high-risk profile) but not for cases with a low-risk profile ($p = 0.493$, Figure 12d).

**IV.4.5. Discussion**

High-grade leukoplakia are generally treated aggressively, often with a wide excision. It is usually agreed that such aggressive treatment reduces the rate of malignant transformation of these high-grade lesions (severe dysplasia and CIS). However, the treatment of leukoplakia with minimal or no dysplasia varies. Some choose to monitor such lesions, some favor removal with a minimal margin, while others advocate more aggressive treatment. Such uncertainty in treatment of low-grade lesions is reflected in this study. While 62% of the cases were treated by clinical removal but with varying width of margins, more than one third were not treated and the decision to treat was not influenced by known risk factors (degree of dysplasia, site and clinical appearance of leukoplakia).

It has been suggested that molecular criteria could complement the available risk indicators and lead to better predictions of progression risk (Califano et al., 1996; Fliss et al., 2000; Lee et al., 2000; Partridge et al., 1998; Partridge, 2000a; Rosin et al, 2000; Westra et al., 1998; Zhang et al., 2000). In a recent study, we used molecular criteria to classify histologically similar lesions into 3 groups with different risks of progression to SCC, with cases in the high-risk group showing a 24 to 33 fold increase in progression risk and more than half of the cases expected to progress within 5 years (Rosin et al., 2000). With these findings, one would expect that treatment impact would be most apparent amongst such cases in the current study; and
conversely, would have minimal effect amongst cases with low-risk genetic profiles, characterized by a low progression rate. Indeed, our results showed a general trend of reduction in the progression risk for each of the 3 risk groups after treatment to remove the clinical lesion although this reduction was not statistically significant in any group examined (low-risk: $p = 0.221$; intermediate: $p = 0.115$ and high: $p = 0.262$, Figure 12).

One explanation for the lack of statistical significance may be the small number of cases in each group. However, a more likely explanation is that although the lesion was judged clinically to be completely removed, the treatment was incomplete. Our data support the latter possibility. When we compared LOH patterns in repeat biopsies for some of these cases, a significant portion of lesions, judged clinically to have been removed, had clonal re-growth of the initial lesion, suggesting that the initial lesion was not totally removed (Figure 10). With this additional molecular information included in the assessment of treatment, lesion removal produced a significant decrease in the progression risk for cases with intermediate- and high-risk genetic profiles ($p = 0.043$ and 0.001, respectively, Table 24, Figure 12). For lesions with the high-risk profile, lesion removal resulted in a 9.1-fold decrease in progression risk. On the other hand, we observed no impact of treatment on cases with low-risk profile. Such data suggest that lesions classified into this molecular subgroup have a progression risk that is so low that there is little or no impact of treatment on outcome.

There have been studies, though limited, showing that molecular means could serve as powerful tools not only to identify which subset of leukoplakia should be treated, but also to judge the efficacy of treatment. Mao et al. (1998) used both clinicohistological and LOH assays to assess the efficacy of N-(4-hydroxyphenyl)retinamide (4HPR) chemoprevention on leukoplakia of the oral cavity and larynx. "Scars" of genetically defective clones could still be
detected in many patients despite morphological remission of lesions, possibly explaining why the lesions reappeared in such individuals after cessation of chemoprevention (Brennan et al., 1995; Hong et al., 1986; Mao, 2000; Westra et al., 1998). These data further support the inclusion of molecular markers in assessing treatment.

Clinical assessment of the completeness of removal is regarded by many to be problematic with histological confirmation desirable. Incomplete removal may explain the reported lack of treatment effect in reducing cancer risk for leukoplakia in some studies leading to the false conclusion that removal was futile (Lumerman et al., 1995; Einhorn and Wershall, 1967). Unfortunately, many clinicians rely only on clinical endpoint and tissue is often not available for histological examination. For example, the increasing popularity of laser ablation therapy, due to its speed and reduced scarring potential, restricts treatment assessment to clinical level. Given the high rate of recurrence of some oral leukoplakia, there is a need for studies aimed at determining a safe margin width that would ensure the complete elimination of a dysplastic lesion especially for those with elevated progression risk.

Despite the inherent problems and limitations associated with the retrospective nature of this study -- e.g., the unavoidable selection bias for cases with clinical history and the incomplete data on treatment details such as margin widths -- our study still suggests that molecular markers could shed light on the very provocative issue of low-grade dysplasia management. Such markers can be use both to define progression risk (Califano et al., 1996; Mao et al., 1996d, Rosin et al., 2000) and to evaluate treatment success. The identification and adequate treatment of seemingly low-risk leukoplakia may be key to improving mortality and morbidity of oral cancer.
Table 21. Clinicopathological features of patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All cases</th>
<th>Progressing</th>
<th>Non-progressing</th>
<th>p-value $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>66</td>
<td>20</td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>Mean age in years (± SD)</td>
<td>55.6 ± 13.9</td>
<td>59.3 ± 12.9</td>
<td>54.0 ± 14.1</td>
<td>0.160</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36/66 (55%)</td>
<td>10/20 (50%)</td>
<td>26/46 (57%)</td>
<td>0.789</td>
</tr>
<tr>
<td>Female</td>
<td>30/66 (45%)</td>
<td>10/20 (50%)</td>
<td>20/46 (43%)</td>
<td></td>
</tr>
<tr>
<td>Histological diagnosis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>33/66 (50%)</td>
<td>7/20 (35%)</td>
<td>26/46 (57%)</td>
<td>0.180</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>33/66 (50%)</td>
<td>13/20 (65%)</td>
<td>20/46 (43%)</td>
<td></td>
</tr>
<tr>
<td>Site of lesion $^b$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-risk</td>
<td>42/66 (64%)</td>
<td>13/20 (65%)</td>
<td>29/46 (63%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Low-risk</td>
<td>24/66 (36%)</td>
<td>7/20 (35%)</td>
<td>17/46 (37%)</td>
<td></td>
</tr>
<tr>
<td>Clinical appearance $^c$:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogeneous</td>
<td>7/31 (23%)</td>
<td>1/13 (8%)</td>
<td>6/18 (33%)</td>
<td>0.191</td>
</tr>
<tr>
<td>Non-homogeneous</td>
<td>24/31 (77%)</td>
<td>12/13 (92%)</td>
<td>12/18 (67%)</td>
<td></td>
</tr>
<tr>
<td>Smoking history $^c$ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>9/58 (16%)</td>
<td>4/18 (22%)</td>
<td>5/40 (12%)</td>
<td>0.438</td>
</tr>
<tr>
<td>Smoker (Ever)</td>
<td>49/58 (84%)</td>
<td>14/18 (78%)</td>
<td>35/40 (88%)</td>
<td></td>
</tr>
<tr>
<td>Follow-up (months)</td>
<td>77.4 ± 48.9</td>
<td>48.5 ± 44.7</td>
<td>90.0 ± 45.5</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

$^a$ p-value for comparison between progressing and non-progressing columns

$^b$ High-risk sites: floor of mouth, ventrolateral tongue, soft palate complex; low-risk site: rest of oral cavity.

$^c$ Smoking habit was available for 58 cases and clinical appearance for 31.
Table 22. Lack of an impact of clinicopathological features on decision to treat premalignant lesions

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All cases</th>
<th>Not Treated</th>
<th>Treated</th>
<th>( p )-value (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cases</td>
<td>66</td>
<td>25 (38%)</td>
<td>41 (62%)</td>
<td></td>
</tr>
<tr>
<td>Histology: Mild dysplasia</td>
<td>33</td>
<td>14 (42%)</td>
<td>19 (58%)</td>
<td>0.612</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>33</td>
<td>11 (33%)</td>
<td>22 (67%)</td>
<td></td>
</tr>
<tr>
<td>Location: High-risk site</td>
<td>42</td>
<td>15 (36%)</td>
<td>27 (64%)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-risk site</td>
<td>24</td>
<td>9 (38%)</td>
<td>15 (62%)</td>
<td></td>
</tr>
<tr>
<td>Appearance (^2): Homogeneous</td>
<td>7</td>
<td>3 (43%)</td>
<td>4 (57%)</td>
<td>0.676</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-homogeneous</td>
<td>24</td>
<td>8 (33%)</td>
<td>16 (67%)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) \( p \)-value for comparison of proportion of cases that were treated among different clinicopathological risk groups.

\(^2\) 31 cases with information available
Table 23. Impact of treatment on progression of dysplasia with different genetic patterns with completeness of removal judged by clinical elimination of leukoplakia

<table>
<thead>
<tr>
<th>Genetic profile</th>
<th>Total cases</th>
<th>Proportion of cases that progress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
</tr>
<tr>
<td>Low-risk LOH pattern</td>
<td>25</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td></td>
<td>(15 treated, 10 untreated)</td>
<td></td>
</tr>
<tr>
<td>Intermediate-risk LOH pattern</td>
<td>15</td>
<td>1/7 (14%)</td>
</tr>
<tr>
<td></td>
<td>(7 treated, 8 untreated)</td>
<td></td>
</tr>
<tr>
<td>High-risk LOH pattern</td>
<td>26</td>
<td>10/20 (50%)</td>
</tr>
<tr>
<td></td>
<td>(20 treated, 6 untreated)</td>
<td></td>
</tr>
<tr>
<td>All cases (n= 66)</td>
<td>66</td>
<td>11/42 (26%)</td>
</tr>
</tbody>
</table>

Risk of progression determined by molecular assessment of initial lesion: low-risk (retention of 3p and 9p); intermediate risk (loss at 3p &/or 9p); high-risk (loss at 3p &/or 9p plus loss at 4q, 8p, 11q, 13q, or 17p).
Table 24. Impact of treatment on progression of dysplasias with different genetic patterns with completeness of removal judged with a combination of clinicopathological and molecular criteria

<table>
<thead>
<tr>
<th>Genetic profile</th>
<th>Total cases</th>
<th>Proportion of cases that progress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Removed</td>
</tr>
<tr>
<td>Low-risk LOH pattern¹</td>
<td>25</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Intermediate-risk LOH pattern¹</td>
<td>15</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>High-risk LOH pattern¹</td>
<td>26</td>
<td>2/12 (17%)</td>
</tr>
<tr>
<td>All cases (n= 66)</td>
<td>66</td>
<td>2/26 (8%)</td>
</tr>
</tbody>
</table>

¹Risk of progression determined by molecular assessment of initial lesion: low-risk (retention of 3p and 9p); intermediate risk (loss at 3p &/or 9p); high-risk (loss at 3p &/or 9p plus loss at 4q, 8p, 11q, 13q, or 17p).
## Figure 10

<table>
<thead>
<tr>
<th>Sample</th>
<th>LOH at specific loci on chromosome arms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DX</td>
</tr>
<tr>
<td>1</td>
<td>MI</td>
</tr>
<tr>
<td></td>
<td>MO</td>
</tr>
<tr>
<td></td>
<td>H</td>
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<td>A</td>
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<tr>
<td>2</td>
<td>MI</td>
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<td>MI</td>
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<td>MI</td>
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<td>CIS</td>
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<tr>
<td>20</td>
<td>MO</td>
</tr>
</tbody>
</table>
Figure 10: Alterations in LOH pattern of 18 loci on 7 chromosomes in progressing dysplasias. The assessment of clonality of sequential biopsies of 20 patients is summarized using color coding. Grey coding indicates loci that show no alteration in LOH status between the initial and subsequent biopsies. Green coding indicates the loss of heterozygosity at a locus in later biopsies of a lesion site. Loci with yellow coding had the same LOH status in the initial dysplasia and the final SCC; however, intermittent biopsies had a different status for that locus. The latter might represent a treatment artifact with later re-growth of subclones present in the initial biopsy. All loci with red coding represent cases in which loci that formerly showed allelic loss in the initial biopsy showed retention of both alleles in both subsequent biopsies and the SCC. Cases were conservatively scored as having insufficient evidence for clonality if greater than 1 of the loci had alterations in LOH status that could not be attributed to the outgrowth the initial dominant clone, i.e. loci coded red. Thus, cases 1 and 5 are considered non-clonal in further analyses. N, non-informative; L', loss of upper allele; L, loss of lower allele; R, retention of both alleles. Blank squares indicates that there was not enough material for analysis or the sample was unavailable for study. Diagnosis (DX) are: MI, mild dysplasia; MO, moderate dysplasia; H, hyperplasia; A, atypia; CIS, carcinoma in situ; and T, SCC. Ti is the time in months between the initial and subsequent biopsy. Primers were 1, D3S1300; 2, D3S1234; 3, D3S1228; 4, IFNA; 5, D9S1751; 6, D9S171; 7, tp53; 8, D17S786; 9, CHRNA1; 10, FABP2; 11, D4S243; 12, D8S261; 13, D8S262; 14, D8S264; 15, D11S1778; 16, INT2; 17, D13S170; 18, D13S133.
Figure 11: Microsatellite analysis of a dysplastic lesion that later progressed to SCC.

Microsatellite markers were used to amplify DNA from normal stroma (C), from cells dissected from initial dysplasia (D1), a recurrent lesion (D2) excised 5 months later and an SCC (T3) diagnosed at the same site six months thereafter. A biopsy of the same lesion taken 36 months before D1 was diagnosed as atypia, but was unavailable for study.

left, Retention of both alleles at \textit{IFNA} for first dysplasia, with loss of the upper allele in the second dysplastic biopsy and the subsequent SCC. 

right, Retention of both alleles at \textit{D8S262} for the first and second dysplasias with loss of the upper allele developing later and present only in the SCC.
Figure 12. Impact of treatment on the probability of having no progression to cancer for dysplasias with different genetic patterns.

Dysplasias were categorized into 3 groups based on molecular patterns: low-risk (retention of 3p and 9p, n = 25); intermediate-risk (loss at 3p and/or 9p, n = 15) and high-risk (loss at 3p and/or 9p plus loss at 4q, 8p, 11q, 13q or 17p, n = 26). A-C) progression as a function of clinical removal of lesion. D-F) progression as a function of removal judged by clinical and molecular (all removal) criteria.
CHAPTER V (PAPER 3).

“COMPARISON OF HPV INFECTION, P53 MUTATION AND ALLELIC LOSSES IN POSTTRANSPLANT AND NON-POSTTRANSPLANT ORAL SQUAMOUS CELL CARCINOMAS”.

Submitted to Journal of Oral Pathology and Medicine
V.1. Thesis summary for manuscript

Transplant recipients show an increased incidence of *de novo* malignancy, and a significant portion of these posttransplant tumors are in the oral cavity. The cause for this increased risk remains unclear. Defective immune function is suspected to be a major contributing factor since immunosuppressive treatment is invariably given to people received solid organ transplant to avoid organ rejection, and frequently given to people received bone marrow transplant (BMT) to alleviate graft versus host disease (GVHD). Few studies have been published on the posttransplant oral SCC. With advances in transplant technology and better management of organ rejection, a steady increase can be expected in the numbers of people with allogenic transplants. As a result, we expect to see more post-transplant cancer including oral cancer and an understanding of the mechanism for these cancers and early identification of these cancers are becoming increasingly important.

The objectives of this study were two-folded. The first was to identify clinicopathological features that might predict the development of posttransplant oral SCC. This was achieved by reporting 5 cases of posttransplant oral squamous cell carcinoma (SCC) with detailed clinicopathological history. The second objective was to elucidate the mechanism underlying the tumorigenesis of posttransplant oral SCC. This was attained by studying and comparing the genetic profiles of the 5 posttransplant oral SCC with 34 SCCs from patients who had received no transplants.

Polymerase chain reaction (PCR)-based assays were used to determine the frequency of allelic loss on chromosomes 3p, 4q, 8p, 9p, 11q, 13q and 17p, and to detect human papilloma virus (HPV) infection. Posttransplant oral SCCs were also analyzed for *p53* mutation.
Archival samples of both posttransplant and non-posttransplant oral SCCs were selected from the Provincial Oral Biopsy Service and Vancouver General Hospitals at Vancouver Hospital & Health Sciences Centre. Tissue sections were cut from the paraffin blocks and microdissected. DNA was quantitated and analyzed for LOH using 19 microsatellite markers 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). Samples were analyzed for HPV DNA using 2 HPV-consensus primers (MY09/MY11, GP5/GP6), and 2 HPV type-specific primers (TS16 and TS18). The analysis of p53 mutation was performed for exons 5-8 (Sidransky et al., 1991; Tseng et al., 1999).

The results showed that the time from transplant to the development of oral SCC is relatively long, averaging 10 years (range 5 to 12 years), signifying a long interval during which intervention would be feasible.

We examined possible clinicopathological features that might predict the appearance of the 5 posttransplant oral SCCs. All 3 patients with BMT had suffered long-term chronic oral GVHD and the posttransplant oral SCC was preceded at the same site by chronic GVHD. In addition to GVHD, mild dysplasia also was present in both available biopsies preceding oral SCC. These findings if supported by later studies would indicate that oral GVHD should be closely followed up for areas suspicious of cancerization and that oral dysplasias if present in these patients should be seriously treated even if the degree of dysplasia is not pronounced.

We investigated possible differences in etiological factors and molecular mechanisms of development of posttransplant oral SCC. Compared to oral SCCs from non-transplant patients,
posttransplant oral SCCs tended to be from males (100% male vs. 53%; \(p = 0.066\)), from significantly younger people (mean 49 years vs. 70; \(p = 0.0007\)) and from people less likely to be smokers (40% smokers vs. 83%; \(p = 0.0721\)). Exposure to alcohol products was not prominent in these patients. Lack of alcohol and tobacco abuse and younger age for posttransplant oral SCCs suggests different risk factors.

This was supported by the results of the molecular study. Only 1 of 5 oral SCCs in transplant recipients had \(p53\) mutation, lower than historical data from SCCs in patients without a transplant (40-50%). HPV was detected in 3 of the 5 (60%) posttransplant oral SCCs, more frequently than 12% found in non-posttransplant oral SCCs (\(p = 0.0346\)). Such results strongly imply a role of HPV in the pathogenesis of posttransplant oral SCC, possibly through inactivation of p53 protein, which was frequently inactivated through \(p53\) mutation in non-posttransplant oral SCC.

A similar frequency of allelic losses was observed in the 2 groups at 3p, 9p, 17p and 8p, but a lower frequency of loss was observed for posttransplant oral SCC in at 4q (39% vs. 0%), 11q (53% vs. 20%) and 13q (45% vs. 20%).

In summary, this pilot study identified distinct clinicopathological features in posttransplant oral SCC that deviated from the patterns observed in conventional SCC. The inverse association between \(p53\) mutation and HPV infection suggests that HPV plays an important role in the development of posttransplant oral SCC although the molecular pathways of these cancers appear to partially overlap with that of conventional oral SCC. These results, if confirmed by future studies, would have important clinical implications in the management of oral lesions of posttransplant patients.
V.2. Objectives

1. To obtain information on HPV infection, allelic loss of regions on chromosome 3p, 4q, 8p, 9p, 11q, 13q and 17p, and p53 mutation in 5 SCCs from transplant patients (TP).

2. To obtain information on HPV infection in 34 SCCs from non-transplant patients (NTP).

3. To use above data (HPV in TP versus HPV in NTP; LOH in TP versus LOH in NTP; and p53 in TP versus p53 in oral SCC from literature) to elucidate possible molecular differences between oral SCC from TP and NTP.

V.3. Hypothesis

1. The genetic pattern (allelic losses) in oral SCCs from transplant recipients is similar to that of conventional oral SCC from non-transplant patients.

2. Decreased immune function and resulting increases in infection by oncogenic virus such as HPV play an important role in the increase in oral SCCs for patients with previous transplants. Hence it is expected that a higher frequency of HPV would be seen in oral SCCs from transplant patients as compared to those from non-transplant patients.
3. One of the activities of the HPV viral proteins is to inactivate the p53 protein. This may reduce the requirement for mutation at this critical gene. Hence if a higher frequency of HPV infection is seen in oral SCCs from transplant patients, a lower frequency of p53 mutation may be seen in these SCCs as compared to those from non-transplant patients.

V.4. Submitted paper

V.4.1. Abstract

Transplant recipients show an increased cancer incidence. The cause for this increased risk remains unclear. A significant portion of these tumors are oral squamous cell carcinomas (SCCs), but few studies have been published in this area. Here we report 5 oral SCCs from transplant patients (TP). To elucidate the mechanism underlying the tumorigenesis, genetic profiles of these 5 SCC were compared with 34 SCCs from non-transplant patients (NTP). Polymerase chain reaction (PCR)-based assays were used to detect human papilloma virus (HPV) infection and to determine the frequency of allelic loss on chromosomes 3p, 4q, 8p, 9p, 11q, 13q, and 17p. TP SCCs were also analyzed for p53 mutation. The results showed that TP were younger (p = 0.0007) and less likely to be smokers (p = 0.07) compared with NTP, suggesting differences in the risk factors. Only 1 of 5 TP SCCs had p53 mutation, lower than historical data from NTP SCCs (40-50%). HPV was detected in 3 of the 5 (60%) TP SCCs, more frequently than 12% found in NTP SCCs (p = 0.0346). A similar frequency of allelic losses was observed in the 2 groups at 3p, 9p, 17p and 8p, but a lower frequency for TP SCC at
4q (39% vs. 0%), 11q (53% vs. 20%) and 13q (45% vs. 20%). In summary, this pilot study identified features in these cases that deviated from the pattern observed in NTP. The inverse association between \( p53 \) mutation and HPV infection suggests that HPV plays an important role in the development of TP oral SCC although the molecular pathway of these cancers appears to overlap that of NTP oral SCC.

**V.4.2. Introduction**

A significant rise in cancer incidence, particularly squamous cell carcinomas (SCCs) has been reported in patients who have received organ or bone marrow transplants (Harris & Penn, 1981; Socie \textit{et al.}, 1998). A considerable number of these SCCs are oral SCCs (17.5% in a large study, Curtis \textit{et al.}, 1997). This increase in cancer incidence rises with increasing years of survival after transplant. The mechanisms underlining such increase remains unclear. Defective immune function is suspected to be a major contributing factor. Immunosuppressive treatment is invariably given to people that receive solid organ transplant to avoid organ rejection, and is frequently given to people that receive bone marrow transplant (BMT) to alleviate graft-versus-host disease (GVHD), a transplant bone marrow induced immunoreaction against host (Curtis \textit{et al.}, 1997; Deeg \textit{et al.}, 1996; Socie \textit{et al.}, 1998). Immune defects resulting from these treatments could render the host cancer prone either by faulty immunosurveillance of genetically abnormal cells or by increased infection with oncogenic viruses (Kinlen 1996). In addition, total body irradiation (TBI) and chemotherapy treatment for the primary bone marrow disease in people that have received BMT could result in DNA damage which in turn could contribute to the cancer increase (Thompson \textit{et al.}, 1994; Curtis \textit{et al.}, 1997). With advances in transplant
technology and better management of organ rejection, a steady increase can be expected in the numbers of people with allogeneic transplants and in survival years for these people. As a result, health professionals expect to see more post-transplant cancer and an understanding of the mechanism for these cancers and early identification of these cancers are becoming increasingly important.

The study of molecular changes in posttransplant cancer may shed light on the molecular pathway and hence pathogenesis of these tumors. One study examined a posttransplant oral SCC for the HPV genome and found HPV 16 DNA in this tumor (Demetrick et al., 1990). Other than this one case study, no study has been done on these posttransplant oral SCCs at a genetic level to our knowledge. Here we report 5 cases of posttransplant oral SCCs. Genetic profiles of these 5 SCCs were compared with 34 oral SCCs from non-transplant patients by assaying for the presence of HPV genome and loss of specific chromosomal regions (loss of heterozygosity, LOH) containing known or presumptive tumor suppressor genes that are frequently lost and play important role in the development of oral SCCs from non-transplant patients (Califano et al., 1996; Field et al., 1995; Mao et al., 1996d; Partridge et al., 2000; Rosin et al., 1997, 2000; Zhang et al., 1997; 1999). The 5 posttransplant SCCs were also analyzed for p53 mutation. The objectives were to determine the potential role of HPV in the pathogenesis of these posttransplant cancer and possible differences in the molecular pathway of these lesions from conventional oral SCCs.

V.4.3. Case reports
Case 1. A 27-year-old man received an allogeneic BMT following induction with chemotherapy and TBI for treatment of his chronic myeloid leukemia (CML) in 1990. One year later, the patient developed GVHD involving the skin and gastrointestinal tract, including oral mucosa. The oral GVHD manifested mainly as mucositis and sensitivity to citric or spicy foods on the left lateral tongue. The severity of his oral symptom fluctuated over the year.

In June 1998, he had a chief complaint of discomfort on his left tongue when eating. At this time, he had not been on immunosuppressives for 2½ years. He had never smoked and used alcohol only on social occasions. On examination, multiple white patches with focal erythema were noted along the left lateral tongue, and minor striations were seen involving the gingiva and cheeks. A biopsy of the left tongue showed mild epithelial dysplasia and chronic GVHD. A topical steroid ointment (Dermovate, 15 grams, tid) was prescribed.

He again was seen in October 1998, and reported continuing discomfort of his left tongue despite use of the steroid. On examination, the mucosal changes of gingiva and cheeks remained unchanged from those seen in June. However, the left lateral and ventral surface of the tongue presented with a 0.8 x 3 cm area of irregular patchy leukoplakia with a 0.5 to 0.8 cm area of intense erythema posteriorly. Biopsy showed an invasive SCC.

In December 1998, the patient received radical treatment for the cancer by hemiglossectomy and bilateral neck dissection. The resection sample showed a deeply invasive moderately differentiated SCC with prominent perineural invasion. The tumor extended to the medial deep resection margin. The tongue mucosa showed patchy squamous dysplasia of variable degree. The tumor was classified as a Stage III lesion (T3N0M0). Post-operative radiotherapy was provided. No recurrence has occurred in two years following treatment.
Case 2. A 40-year-old male received BMT from his sibling after conditioning that included TBI for treatment of CML in August 1990. He was a heavy smoker.

The patient experienced extensive chronic oral mucositis and xerostomia following transplantation. The diagnosis of oral GVHD was made clinically and confirmed histologically (grade 1-2) in April 1991, and this was managed by topical steroid and topical Cyclosporine A. In addition, chlorhexidine was prescribed for his poor oral hygiene, and sialogogue for xerostomia as well as periodic antifungal agents for episodes of oral candidiasis. Over time his oral GVHD fluctuated in severity with partial response to the management.

In 1996, an area of fissured leukoplakia was noted in the lower labial mucosa and biopsy showed a squamous papilloma with mild epithelial dysplasia and GVHD.

In August 1997, a granular leukoplakia was identified again on the lower labial mucosa, and a biopsy showed squamous papilloma with mild epithelial dysplasia and GVHD. In November 1997, the lesion on the lower lip mucosa recurred and presented as a palpable thick leukoplakia. Biopsy of the lesion demonstrated a carcinoma in situ (CIS). The lesion was totally excised and the resection specimen revealed an invasive SCC, classified as stage I (T1N0M0).

Case 3. A 49-year-old male received BMT from his sibling after conditioning including TBI for treatment of his acute myeloid leukemia (AML) in March 1994. He had never smoked and did not use alcohol.

In June 1994, oral examination showed lichenoid striae on the cheeks, dilated minor salivary gland ducts on the soft palate, and reduced saliva volume. A clinical diagnosis of mild oral GVHD was made and no treatment was given.

In September 1998, he reported roughness and sensitivity to hot and spicy foods in the lower lip and cheeks. On examination, lichenoid striaes with mild erythema were noted on the
lower lip, cheeks, and lateral tongue. The symptom was treated with a steroid mouth rinse (dexamethasone 0.4mg/5ml, once daily). In November 1998, the sensitivity had not improved, and in addition, increased erythema was seen on the lower labial mucosa near the vermillion border. The steroid mouth rinse was increased to three times daily.

In March 1999, he reported crusting of the lower lip for several months. Examination showed an area of leukoplakia on the lower lip as well as mild lichenoid changes on the cheeks. A biopsy of the lower lip lesion showed a CIS with moderate dysplasia involving the lateral biopsy margins. Topical bleomycin was applied to the area (5 min/day x 20 days) to obliterate the residual dysplastic changes. In August 1999, examination showed recurrent white patches on the lower lip. Biopsies of left and right lower lip showed CIS at both sites. The white patches were treated by laser ablation. In December 1999, examination identified an irregular leukoplakia in the lower labial mucosa near the left corner of the mouth. The lesion was excised with wide margins and again demonstrated CIS histologically. Recurrence has not been seen at last follow-up in January 2001.

**Case 4.** A 57-year-old male received a kidney transplant for renal failure as a result of rapidly progressive glomerulonephritis in 1990. He was maintained on immunosuppressives (Cyclosporine A, 125 mg bid, Prednisone 12.5 mg alternate days and Azathioprine 50 mg tid). He had quit smoking 18 years earlier and did not drink alcohol.

In December 1995, leukoplakia was noted on the right lateral tongue. The lesion was excised and showed moderate dysplasia with focal severe dysplasia. The changes did not involve the biopsy margins.

In February 1996, a recurrent leukoplakia appeared at the previous biopsy site. The lesion was totally excised and showed areas of severe dysplasia with central CIS.
In February 1998, a leukoplakia occurred on the same site. The recurrent lesion was totally excised and biopsy showed mild dysplasia.

In April 1999, the lesion was back again and the biopsy showed CIS.

In June 2000, another recurrence was noted on the same site and the biopsy showed an invasive SCC, classified as stage I (T1N0M0). An excision with wide margin was performed one month later. No recurrence was seen at a 1-year follow-up.

**Case 5.** A 23 year-old man received a kidney transplant for renal failure as a result of rapidly progressive glomerulonephritis in December 1978. He was maintained on Imuran 50 mg and Prednisone 15 mg once daily. He was a past smoker and drank 2 glasses of wine daily.

After the transplant, he suffered a number of illnesses. In 1979, he developed septic necrosis of both femoral heads and a perforated duodenal ulcer, the management of which included vagotomy and pyloroplasty. In 1983, he was diagnosed with a nonseminomatous embryonal carcinoma of the left testis and treated with a left radical orchidectomy. Following this cancer, the dosage of prednisone was decreased to 5 mg/day for 2 days and 7 mg/day for the 3rd day.

In July 1999, a gingival lesion was identified in the lower right premolar region. Biopsy of the lesion revealed SCC. The cancer was managed by segmental resection of both soft and hard tissue including removal of the involved teeth (44,45,46) and the resection specimen revealed an invasive SCC, classified as stage I (T1N0M0). No recurrence has been identified to date.

**V.4.4. Materials and Methods**
Samples and DNA Extraction. Two sample sets were selected from the Provincial Oral Biopsy Service and Vancouver General Hospital. The first consisted of 5 cases of archival paraffin samples of posttransplant oral SCC (see above 5 cases). The second consisted of 34 oral SCCs from patients without a previous history of transplant. All lesions were microdissected and extracted for DNA as described previously (Zhang et al., 1997).

LOH Assay. DNA was analyzed for loss of heterozygosity (LOH) as described previously (Zhang et al., 1997) by using microsatellite markers (Research Genetics, Huntsville, AL) that mapped to the following 12 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 in regions previously shown to be frequently lost in head and neck tumors and premalignant lesions (Zhang & Rosin, 2001; Poh et al., 2001; Zhang et al., 2001a,b).

P53 mutation analysis. Two segments of the p53 gene were separately amplified by PCR to produce a 750 base pair (bp) fragment encompassing exons 7 and 8 and a 500 bp fragment with exons 5 and 6. Further amplification using nested primers generated DNA fragments containing individual exons (Sidransky et al., 1991; Tseng et al., 1999). Amplifications were performed in a 25 µl volume using a Hybaid thermocycler. Each PCR reaction contained 10% dimethyl sulfoxide (DMSO), 6.7 mM MgCl2, 1.5 mM of each deoxyribonucleotide triphosphate (dNTP), 7 ng/µl of each primer, 0.05 units of Taq polymerase (Life Technologies), and 0.8-1 ng/µl template DNA. Amplified DNA was sequenced using the AmpliCycle sequencing kit (Perkin-Elmer, Branchvurg, NJ) in the presence of appropriate radiolabeled primer. Sequencing reactions products were resolved by electrophoresis on a 7%
denaturing polyacrylamide gel and imaged by autoradiography. All mutations detected were verified in a repeat sequencing experiment. DNA sequence polymorphisms were identified by analyzing in parallel paired normal DNA isolated from connective tissues in the same biopsy.

**Detection of HPV sequences.** HPV status was determined by PCR analysis using 2 sets of universal and 2 sets of virus type-specific primers. The MY09/MY11 universal primer set (Manos et al., 1989) yielded a 448-454 bp fragment, GP5/GP6 universal primer set (Snijders et al., 1990) yielded a shorter fragment of 155 bp located within the MY09/MY11 fragment. The virus type-specific primers set TS 16 and set TS 18 yield a 96 bp and a 115 bp amplified fragment, respectively. The primers are designed to detect 2 high-risk oncogenic HPV strains, HPV16 and 18, in paraffin-embedded tissue samples (Baay et al., 1996). Primers within the GAPDH gene were included in each PCR reaction to generate a 225 bp fragment as an internal control. DNA extracted from human cell lines SiHa (which contains HPV 16) and HeLa (which contains HPV 18); were obtained from the American Type Culture Collection, Rockville, MD and used as positive controls for HPV infection. Negative controls included DNA from human lymphocytes, archived lymph nodes and a mock reaction that included reagents only (no DNA). The PCR products were then diluted in gel loading buffer, separated on a 7% polyacrylamide gel containing urea and formamide, and visualized by autoradiography.

V.4.5. Results and Discussion

Table 25 summarizes the clinicopathological characteristics in the above 5 Case Reports, and Table 26 compares the demographic characteristics of the two groups. As is consistent with the literature (Harris & Penn, 1981; Socie et al., 1998), the time from transplant to the
development of oral SCCs seems to be generally long, ranging from 5-21 years (mean 10 ± 6 years). Thus the process should be readily susceptible to intervention. Compared to oral SCCs from non-transplant patients, posttransplant oral SCCs tended to be from males (100% male vs. 53%; \( p = 0.066 \)), from significantly younger people (mean 49 years vs. 70; \( p = 0.0007 \)) and from people less likely to be smokers (40% smokers vs. 83%; \( p = 0.0721 \)). Exposure to alcohol products was not prominent in these patients. Lack of alcohol and tobacco abuse and younger age for posttransplant oral SCCs suggests different risk factors.

Possible contributing factors for the emergence of the 5 posttransplant oral SCC were examined. Chronic usage of immunosuppressive medications was found in 3 of these 5 patients, including both patients with solid organ transplant (on chronic immunosuppressives to prevent rejection of the transplant) and 1 patient with BMT (on chronic immunosuppressives to control extensive chronic GVHD). In addition, as expected, all three patients with BMT had received TBI (possible DNA damage).

We examined possible clinicopathological features that might predict the appearance of the 5 posttransplant oral SCCs. All 3 patients with BMT had suffered long-term chronic oral GVHD and the posttransplant oral SCC was preceded at the same site clinically by chronic GVHD. Two of the three patients had biopsies from the oral lesions preceding the cancer, and both biopsies confirmed the presence of lichenoid mucositis, a feature consistent with chronic GVHD. Similar findings of GVHD preceding oral SCCs have been reported in several recent publications (Millen et al., 1997; Otsubo et al., 1997; Curtis et al., 1997). For example, one study reported 7 patients with post BMT SCCs (5 oral and 2 skin), and all 7 SCCs were preceded at the same sites by chronic GVHD (Socie et al., 1998).
In addition to GVHD, mild dysplasia also was present in both available biopsies preceding oral SCC. The presence and degree of dysplasia are the histological hallmarks for judging malignant potential of oral lesions. Presence of dysplasia in a high-risk (for malignant transformation) area, even if only at low-grade, generally warrants elimination of the lesion. The two dysplastic lesions here, both at high-risk areas (lateral tongue and lower lip), were not managed at the time. This probably was partly because the dysplastic changes were interpreted as epithelial reactive responses to local intense lichenoid mucositis (not indicating cancer risk) instead of true dysplasia. While reactive atypical epithelial changes are characteristically seen in the case of intense acute inflammation or mixed acute and chronic inflammation, typically near an ulcer, it is less clear whether such reactive changes readily occur in specific lichenoid dermatoses such as GVHD. A recent study has shown that dysplasia in oral lichenoid lesions do signal malignant potential (Zhang et al., 2000). Although the case number here is too small to draw any conclusion, it is an area needing further study. It is possible that dysplasia in GVHD, similar to other oral lichenoid lesions, signals malignant potential and pathologists and clinician should not readily discount the significance of dysplasia in such cases.

Table 27 compares the results of molecular analyses of the two groups. A similar proportion of cases in both groups showed allelic losses at 3p (100% vs. 82%), 9p (60% vs. 76%), 17p (60% vs. 64%) and 8p (60% vs. 50%). Compared to oral SCCs from non-transplant patients, the posttransplant oral SCCs demonstrated lower percentage of losses at 11q (53% vs. 20%), 13q (45% vs. 20%), and particularly at 4q (39% vs. 0%). However, the differences did not reach significance. The lack of differences in these latter chromosome arms may have resulted from the small number of posttransplant oral SCCs reported herein and further studies using a larger number of cases are needed to determine if the two groups differ in allelic loss at
some chromosome arms. Nonetheless, the results suggest that the molecular pathway of posttransplant oral SCCs does overlap, at least partially, with that of conventional oral SCC.

Mutation at $p53$ is a common event in oral SCC. The literature shows that about 40-50% of oral SCCs (from non-transplant patients) demonstrate $p53$ mutation (mainly in the hotspots) (Boyle et al, 1993; Brennan et al, 1995). A lower frequency of $p53$ mutation was found in the posttransplant oral SCCs, with only 1 of the 5 (20%) lesions in this study demonstrating $p53$ mutation at hotspots exons 5-8.

A major difference between the molecular profile of oral SCCs from transplant and non-transplant patients was noted in the rate of HPV infection in the cancer cells. Only 4 of 33 (12%) cases of SCCs from non-transplant patients were HPV positive. That was consistent with a recent study in which 12% (10/84) of oral SCCs were HPV positive (Gillison et al., 2000). In contrast, the majority of the 5 post-transplant oral SCCs (3/5, 60%) in this study showed the HPV genome, which was significantly higher than that of conventional SCCs despite the low number of cases used ($p = 0.0346$). Two of the 3 cases were high-risk HPV (16, 18) and the third did not belong to HPV 16 or 18 and was not further sub-typed. The increased rate of HPV infection could explain the lower rate of $p53$ mutation in these lesions since $p53$ protein could be inactivated by HPV protein through a ubiquitin pathway.

Our results suggest that HPV plays an important role in the development of posttransplant oral SCCs. One obvious explanation for the increased HPV infection in this subset of oral SCCs would be chronic immunosuppression resulting in less immunologic protection against infection. Chronic GVHD, a lichenoid mucositis resulted from a cell-mediated immunological process, could have provided additional local factors for posttransplant carcinogenesis. As an inflammatory lesion, it frequently causes ulceration that could provide an
easier entry for HPV, and it has a higher cell turnover that could heighten the chances of genetic errors caused by a carcinogen. In addition, the cell-mediated local immune process also could participate in the carcinogenesis process through unknown mechanism. One recent study showed that all oral SCCs post BMT demonstrated papillomatous lesions in the cancer area as well as chronic GVHD (dense subepithelial lymphoid infiltrate, (Socie et al., 1998). The result is in support of the hypothesis that chronic GVHD provides a better soil for infection by HPV in immunocompromised patients.

In summary, the results of this study suggest that GVHD may precede posttransplant oral SCCs, and that dysplasia in the presence of GVHD should not be discounted. The molecular analyses showed that the molecular pathway of posttransplant oral SCC overlap at least partially with that of conventional oral SCCs, and that HPV is an important causative agent in posttransplant oral SCCs. If these results are confirmed by future studies, that would have important implications in the clinical management of post-transplant patients, including close follow-up, assessing lesions for the presence of HPV, and possibly new strategies in prevention (e.g. vaccination against HPV) and therapy for these infections (zur Hansen, 2000).
Table 25. Clinical characteristics of patients with secondary oral cancer

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age at the time of SCC (Sex)</th>
<th>Disease/ transplant (years to SCC)</th>
<th>Chronic Immune Suppression medication</th>
<th>Site of oral cancer</th>
<th>Posttransplant oral conditions preceding oral SCC</th>
<th>Pathology report of oral conditions preceding oral SCC</th>
<th>Habit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35 (M)</td>
<td>CML(^1)/BMT(^2) (8 years)</td>
<td>None for the last two years</td>
<td>Left lateral tongue</td>
<td>7 years chronic oral GVHD: sensitivity &amp; white patches left tongue, cheeks and gingiva</td>
<td>Biopsy from left tongue: mild GVHD with mild epithelial dysplasia</td>
<td>Non smoker; social drinker</td>
</tr>
<tr>
<td>2</td>
<td>47 (M)</td>
<td>CML/BMT (7 years)</td>
<td>Cyclosporine A Dapsone</td>
<td>Lower lip</td>
<td>7 years chronic oral GVHD: sensitivity, white patches and granular lesions of oral mucosa including lower lip</td>
<td>Biopsies of lower labial mucosa: papilloma with mild dysplasia &amp; lichenoid mucositis (GVHD)</td>
<td>Heavy smoker</td>
</tr>
<tr>
<td>3</td>
<td>54 (M)</td>
<td>AML(^3)/BMT (5 years)</td>
<td>None</td>
<td>Lower lip and labial vestibule</td>
<td>4 years chronic oral GVHD: sensitivity, white patches &amp; erythema on cheeks and lips</td>
<td>No biopsy was done</td>
<td>Non-smoker &amp; non-drinker</td>
</tr>
<tr>
<td>4</td>
<td>65 (M)</td>
<td>Glomerulonephritis / renal transplant (8 years)</td>
<td>Cyclosporine A Prednisone, Azathioprine</td>
<td>Right lateral tongue</td>
<td>Oral leukoplakia right lateral tongue</td>
<td>Biopsy of right tongue: severe dysplasia (excised)</td>
<td>Non-smoker &amp; non-drinker</td>
</tr>
<tr>
<td>5</td>
<td>44 (M)</td>
<td>Glomerulonephritis / renal transplant (21 years)</td>
<td>Prednisone, Azathioprine</td>
<td>Gingiva</td>
<td>No</td>
<td>No</td>
<td>Past smoker; 2 glasses of wine/day</td>
</tr>
</tbody>
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\(^1\)CML: Chronic myeloid leukemia. \(^2\)BMT: Bone marrow transplant. \(^3\)AML: Acute myeloid leukemia
Table 26. Comparison of demographic characteristics of oral SCC patients with or without transplant

<table>
<thead>
<tr>
<th></th>
<th>Number of cases</th>
<th>Gender (% of male)</th>
<th>Average age (±SD)</th>
<th>% of smokers for patients with known habit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-transplant oral SCC</td>
<td>5</td>
<td>5/5 (100%)</td>
<td>49 (±11)</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>Oral SCC from patients without a history of transplant</td>
<td>34</td>
<td>18/34 (53%)</td>
<td>70 (±12)</td>
<td>24/29 (83%)</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td></td>
<td>0.066</td>
<td>0.0007</td>
<td>0.0721</td>
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Table 27. Molecular analysis of oral SCC from transplant as compared to non-transplant patients

<table>
<thead>
<tr>
<th></th>
<th>HPV</th>
<th>p-53 mutation</th>
<th>Chromosome arm</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>3p</td>
</tr>
<tr>
<td>Case 1</td>
<td>-</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>Case 2</td>
<td>+ (18)</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>Case 3</td>
<td>+ (16, 18)</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>Case 4</td>
<td>-</td>
<td>+ (exon 7)</td>
<td>L</td>
</tr>
<tr>
<td>Case 5</td>
<td>+</td>
<td>+ (exon 7)</td>
<td>L</td>
</tr>
<tr>
<td>Summary results from Post-transplant oral SCC</td>
<td>3/5</td>
<td>1/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>(60%)</td>
<td>(20%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Molecular pattern in 34 oral SCCs of non-transplant patients</td>
<td>4/33</td>
<td>54/129</td>
<td>28/34</td>
</tr>
<tr>
<td></td>
<td>(12%)</td>
<td>(42%)</td>
<td>(82%)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0346</td>
<td>0.6483</td>
<td>0.5736</td>
</tr>
</tbody>
</table>

1 Data on LOH at 3p, 9p and 17p in some of these SCC reported in Zhang et al., 1997.

2 Results from Brennan et al., 1995.
CHAPTER VI.

MORE STUDIES AND GENERAL DISCUSSION
Since this thesis contains discussion from the individual published/submitted papers, no overall discussion will be given for all the study results. However, in the following sections, study results or areas that have not been sufficiently discussed in the above 3 papers because of space limitation will be discussed. In addition, significance of the studies and potential future research directions will be mentioned.

VI.1. Genetic progression model for dysplasia to SCC pathway

The establishment of a working molecular progression model for oral cancer development will have great impact on the study of oral cancer. It will not only provide insight into the molecular pathway and mechanisms of disease progression, but also provide means of staging lesions molecularly according to their molecular risk. As reviewed in Section I.3.7, Califano and associates (1996) were the first to establish a molecular progression model for oral cancer development using LOH data. This was a hallmark paper in that the LOH pattern was examined for the first time in the whole spectrum of oral lesions, from hyperplasia, to dysplasia, to cancer. The major pitfall of the progression model was that it grouped all dysplasia (mild, moderate and severe) into one stage. This may partly be the result of difficulty in obtaining enough samples for each individual degree of dysplasia. Unfortunately, it is well known that low-grade dysplasia has drastically different clinical behavior compared to high-grade premalignant/preinvasive lesions. Only a small percentage of low-grade dysplasia will progress into cancer, while a much higher percentage of high-grade premalignant lesions will progress, hence, low-grade and high-grade dysplasias likely represent different stages of lesions. The lack of molecular information on the early low-grade lesions means that the critical information on
the molecular changes in early dysplasia that may predict cancer risk still is unavailable. In the same way, the lack of molecular information on high-grade preinvasive lesions denotes a lack of genetic information on gene changes signaling invasion.

To address this problem and refine the molecular model, a plan was made to investigate LOH genetic patterns for low-grade dysplasia (mild and moderate), and high-grade dysplasia (severe and CIS) separately in addition to the study of early hyperplastic lesions and later SCC, using 19 primers on 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p). This represents a large endeavor, and parts of the data generated first have contributed to a recent publication from our lab (Rosin et al., 2000, I am one of the authors). This thesis carried out the remaining LOH analyses to complete and refine this model. The raw data were presented in Chapter III, but the objectives of that paper were to investigate the genetic pathway of the verrucous lesions and to compare the verrucous lesions with dysplasia and SCC. As a result, the LOH data on dysplasia and SCC were used for comparison with the verrucous lesions and the genetic progression pathway for dysplasia to SCC pathway was not specially discussed. Here, the refined genetic progression pathway for dysplasia to oral SCC is presented.
Table 28. Allelic loss in reactive hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia/CIS and SCC

<table>
<thead>
<tr>
<th></th>
<th>Hyperplasia</th>
<th>Mild dysplasia</th>
<th>Moderate dysplasia</th>
<th>Severe dysplasia/CIS</th>
<th>SCC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td># of case</td>
<td>47</td>
<td>31</td>
<td>23</td>
<td>38</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Mean loss</td>
<td>0.19 ± 0.4</td>
<td>0.81 ± 1.17</td>
<td>1.70 ± 1.72</td>
<td>2.82 ± 1.61</td>
<td>3.6 ± 1.8</td>
<td>0.071</td>
</tr>
<tr>
<td>Any loss</td>
<td>9/47 (19%)</td>
<td>14/31 (45%)</td>
<td>15/23 (65%)</td>
<td>36/38 (95%)</td>
<td>39/41 (95%)</td>
<td>1</td>
</tr>
<tr>
<td>&gt;1 arm lost</td>
<td>0/47 (0%)</td>
<td>6/31 (19%)</td>
<td>10/23 (43%)</td>
<td>33/41 (80%)</td>
<td>33/41 (80%)</td>
<td>0.786</td>
</tr>
<tr>
<td>3p</td>
<td>5/44 (11%)</td>
<td>6/29 (21%)</td>
<td>7/24 (29%)</td>
<td>21/38 (55%)</td>
<td>29/40 (73%)</td>
<td>0.157</td>
</tr>
<tr>
<td>9p</td>
<td>1/46 (2%)</td>
<td>10/30 (35%)</td>
<td>14/24 (58%)</td>
<td>27/37 (73%)</td>
<td>27/39 (69%)</td>
<td>0.803</td>
</tr>
<tr>
<td>4q</td>
<td>0/43 (0%)</td>
<td>2/28 (7%)</td>
<td>2/20 (10%)</td>
<td>13/33 (39%)</td>
<td>13/39 (33%)</td>
<td>0.630</td>
</tr>
<tr>
<td>8p</td>
<td>0/45 (0%)</td>
<td>2/28 (7%)</td>
<td>6/23 (26%)</td>
<td>9/32 (28%)</td>
<td>1</td>
<td>20/39 (51%)</td>
</tr>
<tr>
<td>11q</td>
<td>2/44 (5%)</td>
<td>4/30 (13%)</td>
<td>2/23 (9%)</td>
<td>6/37 (16%)</td>
<td>6.983</td>
<td>20/40 (50%)</td>
</tr>
<tr>
<td>13q</td>
<td>1/43 (2%)</td>
<td>1/30 (3%)</td>
<td>1/23 (4%)</td>
<td>3/33 (9%)</td>
<td>0.6363</td>
<td>15/38 (39%)</td>
</tr>
<tr>
<td>17p</td>
<td>0/45 (0%)</td>
<td>3/30 (10%)</td>
<td>8/24 (33%)</td>
<td>24/37 (65%)</td>
<td>24/40 (60%)</td>
<td>0.814</td>
</tr>
</tbody>
</table>

1 Value in red indicates significant increase for the arm for the first time. 2 Reactive hyperplasia versus low-grade dysplasia. 3 Low-grade dysplasia versus High-grade dysplasia. 4 High-grade dysplasia versus SCC.
VI.2.1. Low-grade and high-grade dysplasia have different frequency of allelic loss

As shown in Table 28, this study involved microsatellite analysis of a whole spectrum of oral lesions, including hyperplasia (47 cases), mild dysplasia (31 cases), moderate dysplasia (23 cases), severe dysplasia/CIS (38 cases) and invasive SCCs (41 cases) using microsatellite markers on 7 chromosomes. Allelic loss was compared among the different groups both in terms of frequency of losses and pattern of losses.

With increasing severity of the lesions (hyperplasia to low-grade dysplasia to high-grade dysplasia to SCC), there was significant increase in the frequency of LOH ($p < 0.0001$, Figure 13). Although allelic loss was sometimes present in hyperplastic lesions (19% show LOH at 1 of 7 arms), the number of lesions with such loss was lower than in mild dysplasia (45%, $p = 0.0217$). In addition, the loss in hyperplasia was limited to a single chromosome arm, with multiple losses occurring only in dysplastic lesions and SCCs.

Figure 13. Histogram of frequency of allelic loss in Dysplasia/SCC pathway

When the frequency of LOH was compared between low-grade and high-grade dysplasia, high-grade dysplasia almost always demonstrated allelic loss (95% of cases had loss on at least
one arm), which was significantly higher than mild and moderate dysplasia together (59%, $p < 0.0001$), and higher than moderate dysplasia (65%, $p = 0.0042$). There were significantly increased multiple losses from low-grade dysplasia to high-grade dysplasia (31% vs. 76%, $p < 0.0001$). Likewise, there were continuing increases in the frequency of allelic losses from high-grade dysplasia to SCC, and the difference in the mean number loss between high-grade dysplasia and SCC was approaching significance ($p = 0.071$).

VI.2.2. Low-grade and high-grade dysplasia have different pattern of allelic loss

In hyperplasia, the most prevalent early change was 3p loss, occurring in 11% of these lesions. Losses on the other arms were rare (ranging from 0 – 5%) in hyperplasia.

In mild dysplasia, there was a significant increase in the frequency of LOH at 9p (2% in hyperplasia versus 35% in mild dysplasia, $p = 0.0003$). Although loss at 3p was the second most common in the mild dysplasia, no significant difference in 3p loss between hyperplasia and low-grade dysplasia existed, reflecting the fact that loss at this arm was the most frequent loss seen in hyperplastic lesions.

Formation of moderate dysplasia was accompanied by a significant increase in LOH at 17p (10% in mild dysplasia vs. 33% in moderate dysplasia, $p = 0.0459$) and a continuing rise in LOH at 9p (35% in mild dysplasia vs. 58% in moderate dysplasia, approaching significance: $p = 0.0987$).
Formation of preinvasive lesions (severe dysplasia and CIS) was accompanied by a significant increase in LOH at 4q (10% in moderate dysplasia vs. 39% in severe dysplasia and CIS, \( p = 0.0284 \)), and continuing increase in LOH at 17p \( (p = 0.0201) \) (Figure 14).

**Figure 14. Histogram of Pattern of Allelic Loss in Dysplasia/SCC Pathway**

\[ * p < 0.05, \text{compared to lower 1 degree of histology group} \]
With the advent of invasive SCC, significant increases were noted for LOH at 11q (16% in severe dysplasia/CIS vs. 50% in SCC, \( p = 0.002 \)) and 13q (9% in severe dysplasia/CIS vs. 39% in SCC, \( p = 0.005 \)), with 8p of marginal significance (28% in severe dysplasia/CIS vs. 51% in SCC, \( p = 0.057 \)). From these results, a genetic progression model for oral cancer can be proposed (Figure VI-3).

**Figure 15. Genetic progression pathway for dysplasia to SCC pathway**

In summary, the study results showed that accumulation of genetic changes (number of changes or hits, or number of allelic losses) was strongly associated with risk of progression to cancer, and there also appeared to be a preferred pattern by which a majority of the lesions may progress: LOH at 3p was the earliest event, associated with transition from normal to benign hyperplasia; LOH at 9p was the second earliest event, associated with transition from hyperplasia to mild dysplasia; Loss at 17p raised with transition from mild to moderate dysplasia, and this
was followed by LOH at 4q from transition of moderate dysplasia to preinvasive high-grade lesions (severe dysplasia and CIS); Invasion and formation of SCC was characterized by additional deletions on 8p, 11q and 13q.

Although rudimentary, this genetic progression model for oral cancer provides a framework for the study of the independent steps involved in this multistep process. This also may be a foundation for the establishment of a risk model for oral premalignant lesion and oral cancer.

With this genetic progression pathway, we are now in a better position to understand the significance of the pattern of losses. As mentioned before, a study from this lab has shown that LOH at 3p &/or 9p seems to be an essential step for cancer progression; those lesions with LOH at 3p &/or 9p had a 3.8 fold increase in relative cancer risk compared to lesions without such losses, whereas those lesions with additional losses (at 4q, 8p, 11q, or 17p) showed a 33-fold increases in relative cancer risk. These 3 risk groups correspond to the sequence of allelic loss depicted in the genetic progression pathway nicely. That is, loss at 3p and 9p are the earliest and essential events, and additional losses on the other arms are later events and indicate higher molecular risk.

VI.2. Genetic progression pathway for VH to VC pathway

Unlike the gradual allelic losses with increasing dysplasia in the dysplasia to SCC pathway, the progression from hyperplasia to VH was shown to be accompanied by sharp rises in
losses in multiple chromosomal arms (9p, 4q, 8p, 11q, 13q, and *CHRNBI*). The progression from VH to VC corresponded to further increases in LOH at 9p and 4q (Figure 16).

With this genetic progression pathway, we are now in a better position to understand the high cancer risk of these verrucous lesions from early stage on as initiation of these lesions seems to be accompanied by rapid acquisition of molecular abnormalities. Consequently these lesions grow relentlessly and are more or less doomed to become cancer.

**Figure 16. Genetic progression pathway for VH/VC pathway**

Normal

\[\text{Hyperplasia} \quad 3p\]

\[\text{Verrucous hyperplasia} \quad 9p, 4q, 8p, 11q, 13q, CHRNBI\]

\[\text{Verrucous carcinoma} \quad 9p, 4q\]
VI.3. HPV, $p53$ and Verrucous lesions

In the first paper (Chapter III. A HIGH FREQUENCY OF ALLELIC LOSS IN ORAL VERRUCOUS LESIONS MAY EXPLAIN MALIGNANT RISK), the verrucous lesions, including both VH and VC, were assayed for allelic loss at 19 loci on 7 chromosomes, and the data were compared with those of hyperplasia, dysplasia and SCC. One of the study results was that the verrucous lesions showed strikingly lower frequency of LOH at 17p (near or in the $tp53$ locus) compared to those of dysplasia and SCC (25% in VC vs. 60% in SCC, $p = 0.037$). Of the verrucous lesions with LOH at 17p, only 1 showed LOH involving $tp53$ locus although 7 involved $CHRNB1$ (two primers were used for 17p, one for $tp53$, another for $CHRNB1$). The two genes are located tightly together. As $p53$ protein inactivation is regarded as essential for tumorigenesis to occur, one could presume that $p53$ protein in the verrucous lesions is inactivated by other mechanisms. One well known ‘other’ mechanism in the inactivation of $p53$ protein is through infection with oncogenic HPV. The protein of HPV could both bind and degrade $p53$ protein through a proteolytic cell system known as the ubiquitin pathway (Neil et al, 1997). Combined with the fact that the verrucous lesions show clinicopathological features highly suggestive of HPV infection, and the fact that they contain low LOH at 17p, we could hypothesize that $p53$ protein in the verrucous lesions is inactivated by HPV protein through a ubiquitin pathway instead of through $p53$ mutation and chromosome losses, resulting in a lower rate of LOH at 17p in these lesions.
The effect of TSGs is autosomal recessive, and according to Knudson’s hypothesis (1985), both copies of a tumour suppressor gene have to be inactivated for its protective function to be lost in a cell. Experience with known suppressor genes suggests that this inactivation 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. If one presumes that p53 protein is inactivated by other mechanisms such as HPV protein, then not only should there be low LOH at 17p, but also a low rate of p53 mutation. The study of the HPV genome in verrucous lesions could provide direct evidence of whether HPV plays a role in the tumorigenesis of verrucous lesions.

To prove our hypothesis, a pilot study was performed after the first paper (Chapter III) to investigate the frequency of p53 mutation and HPV infection in verrucous carcinomas.

VI.3.1. Objectives and hypotheses

1. To investigate p53 mutation in 17 cases of VC by sequencing the hotspots from exons 4 to 8.

   It is hypothesized that p53 protein in verrucous lesions is inactivated by mechanisms other than point mutation of one allele and LOH at the other.
2. To investigate HPV infection in 17 cases of VC by PCR-based analysis of HPV genome using 2 universal primers (MY09/MY11, and GP5/GP6) and 2 virus type-specific primers (TS 16 and TS 18).

It is hypothesized that a high frequency of HPV infection will be found in VC.

VI.3.2. Results and discussion

None of the 17 VC showed \( p53 \) mutation in the hotspots assayed. This result and the previous finding of low rate of LOH at 17p (only one of these involved \( tp53 \)) in these lesions support the hypothesis that \( p53 \) protein in verrucous lesions is inactivated by other mechanism rather than the common pathway of point mutation at one allele and LOH at the other.

Uterine cervical cancer best exemplifies inactivation of \( p53 \) protein through other mechanism without LOH at 17p and without \( p53 \) mutation. Results from cervical cancer on LOH at 17p and \( p53 \) mutation are remarkably similar to our findings in verrucous lesions. In cervical cancer, the frequency of LOH at 17p has been reported at a low 24% (compared to 20% in VC) and most often LOH at 17p does not affect the \( tp53 \) locus (Fujita \textit{et al.}, 1992; Kim \textit{et al.}, 1997; Park \textit{et al.}, 1995; Kersemaekers \textit{et al.}, 1998). In parallel, cervical cancer also shows a low rate of \( p53 \) mutation (less than 10%) (Fujita \textit{et al.}, 1992; Castren \textit{et al.}, 1998; Helland \textit{et al.}, 1998). The mechanism of \( p53 \) inactivation in the cervix is well known through HPV infection.
It has been shown that virtually all cervical cancer is infected with HPV, indicating this is an essential step for cervical carcinogenesis.

However, only 1 of 17 (6%) VC showed HPV infection (type 18). Could this result be a technical failure or artifact? As the positive controls (cell lines known to be positive for HPV16 or HPV18) were positive under the experimental condition, and as the percentage of HPV infection in SCC which were stained together with VC was similar to a recent report from a reputed laboratory at Johns Hopkins, we have ruled out technical failure. If the possibility of technical failure/artifact is ruled out, such result could be interpreted in two ways. First, the inactivation of p53 protein in verrucous lesions does not involve HPV infection but some other unknown mechanism. Alternatively, HPV infections are in these verrucous lesions but the type of HPV is undetectable with current techniques. Traditionally, HPV is believed to play a vital role in carcinogenesis of anogenital cancer. Recently, increasing data show that HPV may also play important role in cancer development in other organs, and these oncogenic viruses may play a much more extensive role in human cancer development than we currently realize. For example, HPV has not been regarded important in the development of skin cancer. Recent advances in technology, however, have shown new evidences indicating certain novel types of HPV are frequently found in skin cancer (Berkhout et al., 2000; Shamanin et al., 1996). It is therefore possible that the failure of identification of HPV in verrucous lesions results from technique limitation.
VI.4. Significance of the research

Oral SCC is believed to develop from a multistep process from hyperplasia to different degree of dysplasia, CIS and finally invasive cancer. Once invasive cancer is formed, the prognosis is poor with 5-year survival rate only about 50%, one of the worst prognoses among major human cancers, and has not been improved in the last several decades despite refinement of surgical techniques and adjuvant therapies. These data have led to an intense interest in developing molecular markers that can be used to genetically characterize high-risk premalignant lesions.

However, the study of molecular changes of oral premalignant lesions has been challenging, and studies on these lesions are few with those done frequently used either a small number of cases or without separating different degree of dysplasias. The difficulty possibly has resulted from several factors: (1). The major research hospitals have ready access to oral SCC but frequent not oral dysplasia, which are commonly biopsied at dentists' office instead of major hospital, and sent to private laboratories. (2). Oral premalignant lesions are usually small and only a small amount of DNA could be obtained. This excludes many samples to be used, and for those with reasonable sizes, the DNA amount is still too small to be used by many molecular techniques. Even for those few techniques could be applied, the requirement for accuracy and precision is high. (3). Microdissection of dysplasia demands a much better pathology knowledge because differentiation of dysplasia from adjacent nondysplastic epithelium is a much harder task than differentiation of overlying oral epithelium from invasive tumor islands/masses.
A major focus of this thesis was to study genetic pathways of oral cancer development with the emphasis on the risk determinants driving the early stage of carcinogenesis. In this thesis, the study on verrucous lesions is the first study on the molecular changes of these lesions, and data obtained have allowed us to propose the first genetic progression model for verrucous hyperplasia to verrucous carcinoma pathway, and a better understanding of the relentless nature of these lesions even from early stage on. This thesis has also provided data to refine the genetic progression pathway for dysplasia to SCC pathway, and for the first time provided information on molecular changes involving different degree of dysplasias. The understanding of the early molecular changes of oral cancer development not only gives us the insight on the mechanism of oral carcinogenesis but also provide tools to specifically target the high-risk premalignant lesions and could have tremendous impact on the management of oral premalignant lesions.

In fact, a study in this thesis has been done to test the hypothesis that lesions with different molecular risk profiles would respond to treatment differently and hence could be managed differently. Our hypothesis was supported by the results from this study, which is the first of its kind.

In addition, the information obtained here could also be applicable to other organs. SCC is the most common human cancer, and occurs in many other organs (e.g., uterine cervix, lung, skin, esophagus, and other head and neck areas). Oral SCC model has been an excellent model for studying SCC throughout the body because of its easy accessibility and easy monitoring of clinical lesions (e.g. leukoplakia). The study of oral cancer development has played a premier role in the study of SCC and has become a paradigm for similar studies of epithelial cancers in other sites (Hong et al., 2000).
The contribution of our research team including the studies in my thesis to the establishment of molecular progression models and risk prediction for oral cancer development is recognized internationally. A recent editorial from the New England Journal of Medicine (Lippman & Hong, 2001) has summarized the latest advances on genetic studies of oral premalignant lesions. The editorial cited 8 significant studies and two of the studies are from our lab (Rosin et al., 2000; Zhang et al., 2001a). The first quoted study (Rosin et al., 2000) involves the risk prediction of low-grade oral dysplastic lesions and also data produced from CHAPTER III (PAPER 1). The importance of our genetic pathway (dysplasia to SCC) is emphasized by the fact that the Editorial has illustrated the molecular progression of oral premalignant lesions by using the study results of our data. The second study quoted by the Editorial involves CHAPTER IV (PAPER 2) in this thesis.

In summary, the studies in my theses are difficult translational studies because these studies involve a wide scope of issues in addition to the laboratory bench work. However, such studies also are very exciting and rewarding because of the potential big clinical impact of the research. I hope that the molecular information obtained in this thesis will contribute to the early identification of high-risk lesions and prevention of oral cancer, as well as SCC in other sites.

VI.5. Ongoing and future researches
VI.5.1. Exfoliative cells as a source of DNA for studying molecular changes of oral premalignant and malignant lesions

The studies in this thesis involved molecular analysis of microdissected samples from biopsy. While the information produced from these studies is highly valuable, improvement in the technique is needed before it can move out of the research laboratory and become readily clinically applicable. One major stumbling block for ready clinical usage of the technique is the process of microdissection. Microdissection is an extremely time-consuming procedure. Generally one person can dissect only two cases each day if each case has only one tissue block and is not particularly complicated. For cancer cases with islands of cancer cells mixed with connective tissue, one may not even finish a single case in a day. Such a tedious and labor-intensive process prevents its usage in clinical settings.

One solution to this problem is to improve the sensitivity of the molecular assay so that it could be used to analyze a small amount of DNA, which could be derived from either exfoliative cells or from quick microdissection of one slide instead of the current practice of dissecting about 15 slides/tissue block.

A major advantage of exfoliative cells is that these could be obtained non-invasively, such as from sputum, saliva or by scraping oral mucosa surface. A scrape, for example, from a premalignant lesion would generally contain pure lesional epithelial cells, hence skip the time-consuming procedure of microdissecting lesional epithelial cells from the underlying connective tissue. The noninvasive nature of obtaining exfoliative cells would allow repeated sampling of a lesion over time. This would be particularly advantageous for follow up of a high-risk lesion.
molecularly over time, not only providing information on the dynamics of the abnormal molecular clone (temporal changes), but also allowing close monitoring of the lesion.

Few studies, however, have used exfoliative cells to study genetic changes, as the amount of DNA obtained from exfoliative cells of a lesion is typically small, precluding the use of many molecular techniques.

Our research team is actively researching the use of exfoliative cells as a source of DNA for molecular analysis. A preliminary study from the lab has shown that molecular results obtained from microsatellite analysis of exfoliative cells is comparable to results from biopsy samples taken immediately after scraping from the same lesion (Rosin et al., 1997). Currently a NIH funded project is using exfoliative cells as a source of DNA for molecular analysis to follow up patients with high-risk oral lesions. I have been actively involved in this longitudinal project, which will still take several years before we see results. Exfoliative oral samples obtained from this perspective study not only provide cells for microsatellite analysis, but also cells for further analysis when our technique is improved in sensitivity.

VI.5.2. Identification of genetically susceptible people for oral cancer development

The major etiological factors for oral cancer include use of tobacco, alcohol, and oncogenic virus. Currently there is increasing interest in the role of genetic susceptibility in the development of cancer. People do not have identical genetic backgrounds. For example, some are born with better metabolic machinery and for DNA repair capacity. Such individual could clear carcinogens more efficiently so that even with carcinogen exposure, their DNA will sustain
less damage or more quickly repaired hence reducing the number of mutations induced. This phenomena may explain why not all smokers get cancer. People with defects in genetic components of these processes would be genetically susceptible to cancer development. For example, people with defects in genes coding for enzymes involved in carcinogen metabolism or with defects in genes coding enzymes for DNA repair would be prone to cancer even with a limited exposure to carcinogen.

Currently one ongoing project of the research team is the study of polymorphisms of genes coding for enzymes in the metabolism of aromatic amine (one type of carcinogens in tobacco). This is a research area that would be valuable to extend into. We would be able to better predict prognosis of an oral premalignant lesion and hence prevent oral cancer by not only knowing the genetic profile of the lesion, but also the genetic profile of the patient.

In addition, I have been collecting oral premalignant and malignant lesions from apparently susceptible patients, for example, young people who have never smoked. In the future, I would like to use these samples to investigate the genetic profiles of these lesions to determine whether they use different genetic pathway for cancer development compared to conventional oral cancer.

VI.5.3. Molecular monitoring of oral cancer patients for cancer recurrence or second primary cancer

The five-year survival rate of oral SCC remains one of the worst among major human cancers (5 year survival < 50%). The major cause for this high mortality rate is a high rate of
local regional recurrence and second primary cancer. Studies show that over 90% recurrence occurs within the first two years (Dhooge et al., 1998). Currently patients with a history of oral cancer are actively followed to monitor for cancer recurrence at a previous cancer site or second primary cancer. However, radiation or surgical therapy results in residual tissue effects in the previous cancer site that could resemble oral premalignant lesions clinically and histologically. As a result, the diagnosis of recurrent cancer or second primary cancer are still frequently delayed.

My thesis has been dealing with molecular analysis of primary oral premalignant and malignant lesions. Molecular markers that could identify high-risk primary oral premalignant lesions could, however, also be of use in the follow-up of cancer patients after treatment to assist in the early identification of high-risk premalignant lesions before they become recurrent tumors or secondary primary tumors. A current ongoing study in our laboratory involves both retrospective and perspective studies of LOH patterns of oral leukoplakias from previous cancer sites to determine whether the molecular risk patterns observed in primary leukoplakia could be of cancer predictive value in these patients. I have actively participated in these projects.

VI.5.4. Molecular markers as powerful tools in the help of making treatment decision and evaluation of treatment

Currently there is no guideline on how to treat low-grade oral premalignant lesions. Some choose to observe, while others prefer treatment. Even those who agree that treatment is necessary do not agree on what the treatment should be. One of the major potential outcomes of
molecular studies such as those in this thesis will be to provide the clinician with an improved ability to target intervention specifically to high-risk lesions. The pilot study in Chapter IV (paper 2, IMPACT OF LOCALIZED TREATMENT IN REDUCING RISK OF PROGRESSION OF LOW-GRADE ORAL DYSPLASIA: MOLECULAR EVIDENCE OF INCOMPLETE RESECTION ) supports this possibility. As this study is the first of its kind, it has been cited in the recent editorial from New England Journal of Medicine (Lippman and Hong, 2001). Because this is an extremely important area, further studies using larger number of patients in prospective trials would certainly be needed.

VI.6. Ending remark

There are many exciting areas we could get into in the study of oral premalignant and malignant lesions. For example, the use of gene microarrays and protein arrays to project the expression changes that characterize for oral premalignant and malignant lesions, novel type of treatment including chemoprevention, approaches to clinical staging of oral premalignant lesions (e.g. size, site, and appearance of a lesion), modeling for the effects of patient life style (e.g. diet and exercise) on cancer development, temporal studies on dynamics of molecular changes over time in dysplastic lesions, etc. With an increasing amount of information available, multilevel modeling of oral cancer development will be needed. This modeling system will consider multiple factors before predicting cancer risk of oral lesions. This information would guide appropriate treatment of these lesions before they become invasive, and lead to prevention of oral cancer.
BIBLIOGRAPHY


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15, 415-25.


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APPENDICES.

METHOD DEVELOPMENT
A.1. Technique development for detection of HPV DNA

During the development of the assay for detection of HPV DNA by polymerase chain reaction, a few technical details need to be worked out. The following sections review these problems.

A.1.1. Positive and Negative control

Inclusion of sufficient negative controls, preferably both with and without DNA, is important in order to detect any possibility of false positive results, arising from contamination or lack of primer specificity. The PCR procedures for the detection of HPV DNA detection are notoriously vulnerable for contamination. This contamination could happen at many steps during the experimental procedures, such as cross-contamination from sample processing (cutting, dissecting, extraction and aliquoting or pipetting). These steps should be handled with extreme caution. In this assay, we used reagents of PCR master mixture without DNA, DNA extracted from fresh human lymphocytes and archived lymph node tissue as HPV negative controls. As we know that HPV is epitheliotropic and will only exist in the epithelial cells. Blood sample or lymph node will become a good source for negative control. The reason I chose archived lymph node DNA as one of the positive control is the samples in our study are all archived formalin-fixed paraffin-embedded tissue. This will make both tested DNA and control
DNA from the same condition. Every 10 to 20 samples inserted one negative control to eliminate the possibility of cross-contamination during the procedure.

Likewise, positive controls should be added to eliminate the possibility of false negatives during the polymerase chain reaction. In the study, the DNA extracted from SiHa and HeLa cell lines were used as positive controls of both consensus primers (MY09/MY11 and GP5/GP6) and type specific primers (HPV16 and HPV18), since SiHa cells contain 1 copy of HPV 16 per cell and HeLa cells contain 10 to 50 copies of HPV 18 per cell. These two cell lines were obtained from the American Type Culture Collection, Rockville.

A.1.2. Multiplex reaction

The samples we used are archived paraffin embedded materials. The possible DNA truncation might happen during the fixation and embedding. To eliminate any non-amplifiable samples and any possible inhibitor remaining in the extracted samples, an additional PCR should be carried out, directed against a DNA sequence present in all cells, e.g., in the gene coding for the human leukocytic antigen (HLA) or house keeping genes. In my study, we used several pairs of house keeping genes (GAP1 and GAP2; GAP1 and GAP3; GAP1 and GAP5) as an internal control of DNA quality. The primers chosen depends on the size of HPV PCR products. For example, the size of the PCR product of the bigger consensus primer (MY09/MY11) is 450 base pairs (bp). We chose GAP1 and GAP4 as the paired primers, since the size of PCR product is 421bp. For smaller sized product like HPV18 (115bp), we chose GAP1 and GAP3 as the paired primers (163bp). To choose similar sized primers will reduce the possible preferential
amplification of the smaller products in the multiplex reaction. This procedure can also serve as an internal control for each polymerase chain reaction to rule out the possibility negative signal from failure of PCR.

A.1.3. Primers chosen and designed

The genome of the HPV types is each unique, yet they share interspersed regions of DNA sequence homology, particularly within the open reading frames E1 and L1.

A mixture of oligonucleotides varying in base sequence but with the same number of bases can be substituted for defined sequence oligonucleotides as primers and successfully coupled with PCR, resulting in the desired gene-specific amplification products. This kind of primers was called degenerate primers. This strategy has been used to design MY09/MY11, one of the consensus primers in HPV DNA detection. This paired consensus primers promote the amplification of an approximately 450bp product from at least 25 distinct HPV types with highly conserved L1 region (see primer Table 12). MY09/MY11 primers are degenerate in several positions, at which one or more possible nucleotides is inserted during synthesis of the oligonucleotide, to render them almost completely complimentary to commonly found HPV, including HPV 6, 11, 16, 18, and 33 etc (PCR protocol, 1990). Another consensus primer chosen is GP5/GP6 primers, which will produce a 155bp product. This is nested to MY09/MY11 and much smaller than MY09/MY11 primers, which might be too big for the paraffin-embedded tissue samples.
In summary, degenerate or non-degenerate and size of the primer also have an impact on the detection rate. Moreover, most HPV consensus primers are primarily designed to detect the HPV types common in cervical samples. Then the failure of detection of HPV in other organs may have resulted from wrong primers used instead of lack of HPV DNA. For example, in the past, HPV was not regarded to be important in cutaneous carcinogenesis. As mentioned above, however, recent studies have suggested that HPV infection could play important roles in cutaneous cancer development by the demonstration of new types of HPV in these lesions. Similarly other types of HPV may play a role in oral carcinomas, and the low detection rate may again be a result of using wrong primers which are limited in cervical cancer. With improvement in technologies, and discovery of new HPV, we may have better picture on the role of HPV in oral carcinogenesis.

For every new primers used, a temperature test and a cycle test were necessary to maximize the PCR reaction and, at same time, to avoid non-significant products formation. To avoid false positive result, decreasing the number of PCR cycles and increasing the annealing temperature were used to increase the stringency of the reaction.

A.1.4. Reading the signals

Several types of signals could be found on the gel:

1. No signal or weak signal on GAPDH gene product position. This means either failure of PCR reaction, poor or truncated DNA template or possible existence of inhibitor. A
second PCR will be performed. If a weak signal of GAPDH gene was found, I will increase the concentration of template DNA to increase the quality of DNA. If this situation cannot be improve by repeat PCR or increase the DNA concentration, this sample will not be qualified for the HPV DNA detection.

2. Positive on GAPDH product but negative on HPV signal. There are three possibilities for this: a. no HPV DNA (true negative); b. low titer of HPV DNA; c. integrated HPV DNA which cannot be detected by consensus primers.

3. Positive on GAPDH product and positive on HPV signal: there are two possibilities for this: a. HPV do exist (true positive); and b. possible contamination.

4. The general consensus primer PCR positive samples were negative by TS-PCR designated as HPV X, indicating the presence of an HPV type different from HPV 16 and 18.

5. The consensus primer negative samples were positive by TS-PCR indicating the possible integration of HPV E6 region in the human genome.

A.1.5. Specificity
DNA extracted from HeLa and SiHa cell lines were used to as positive controls. PCR products of them were cut from gel and sequenced by direct sequencing with one of the paired primers to confirm the existence of HPV sequence.

A.1.6. Possible problems remained

1. Frozen and scrapings were equally suitable for HPV testing. By comparison, HPV analysis of paraffin-embedded materials was much less efficient. The data showed HPV detection rate to be highest in DNA from frozen tissue (58%), and only slightly lower in scrapings from the tumor surface (50%). In paraffin-embedded tissue samples, however, HPV DNA was detected at a much lower rate (17%).

2. Different primers designed, ex: different targeting spots. The possible reasons to explain the different results when comparing oral cases with genital lesions. For example, consensus primers, designed primarily for genital lesions, might not be as efficient for HPV detection in oral cell and tissue material. Most oral HPV infections are probably latent/low productive infections. A previous study pointed out that oral samples generally produced faint PCR products on the gel or were even negative prior to hybridization in contrast to genital specimens run in the same experiment, even frozen or fresh samples used (Ostwald 1994).
3. Although HPV infection occurs in a substantial proportion of oral carcinoma, as repeatedly tested with different techniques including PCR, the role of HPV in carcinogenesis remains undefined. As shown by southern blot analysis, HPV DNA probably often resides in episomal form in oral carcinoma, in contrast to cancer of the cervix uteri. The molecular sequences discussed for cervical carcinoma — virus integration, deletion of viral suppressor genes, activation of viral cancer genes — have not been proven to function in oral cancer. On the other hand, the oral mucosa is directly exposed to many carcinogenic agents, some of which are unequivocally relevant to oral carcinogenesis.

A.2. p53 mutation analysis

In the refinement for p53 mutation analysis, a few technical details need to be pointed out:

1. This to be the most reliable method since other screening modalities, such as RNAases protection (Nishisho, 1991), SSCP (single-strand conformation polymorphism, Orita M, 1989), or denaturing gel electrophoresis (Shirasawa, S, 1991), can miss certain mutation. Because only a small percentage of mutations occur outside the conserved regions, we did not sequence all the exons within p53 (Hollstein, 1991).
2. One amplification, without second nesting PCR, was needed before direct sequencing for exon 5.

3. The genome DNA was archived formalin-fixed and paraffin-embedded material. They might be different from fresh frozen samples in two ways: the amount of DNA is usually less (in quality) and the DNA is more fragmented. This is also the reason that the initial p53 amplification step requires more primers to span a region. In this case, we use 2 primer pairs to span exons 5 and 6 and exon 7 and 8 separately and use nesting primers to reamplify for each exon.

4. To rule out the possible contamination of other DNA source or nonsignificant amplification of primers, a negative control with reagent only was included.

5. To reduce nonsignificant reaction, we can make sure every procedure was undergone in low temperature. A hot start (at 95 degree) to preheat PCR machine is useful to reduce non-specific primer annealing and extension during temperature rising gradually.

6. To make sure the proper amplification, a checking procedure of PCR product has to be been done before further procedure. 3ul of PCR samples mixed with 1ul 10 x loading buffer were loaded onto a 1.5% agarose gel with 1KB DNA ladder. After ethidium bromide staining, bands can be checked under UV light. With this procedure, we can make sure we have the right sized amplification for each PCR. If the band was weak and
diffused, it might indicate that the template DNA was fragmented or the reaction failed.

Either re-PCR or higher concentration of template could be tried to amplify again.