ALLELIC IMBALANCE AT 11Q IN ORAL CANCER AND

PREMALIGNANT LESIONS

by

DING AN

B.Sc., Lanzhou University, China, 1998

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER Of SCIENCE

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THE FACULTY OF GRADUATE STUDIES

(Department of Oral Biological and Medical Sciences; The Faculty of Dentistry)

We accept this thesis as conforming
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Date _May 6, 2002._

ABSTRACT

Oral squamous carcinomas (SCC) remain a significant public health problem worldwide despite advances in therapy and local disease control. New innovative strategies must be developed for the prevention, early detection and treatment of oral carcinoma. Such approaches will be heavily dependent on a better understanding of the molecular mechanisms underlying carcinogenesis at this site.

This thesis describes a series of studies done on oral cancers and premalignant lesions to better define the role of alterations on chromosome 11 in the development of oral cancer at this site. Although numerous studies have reported the presence of alterations on this chromosome arm in oral cancers, few studies have examined premalignant lesions in order to determine whether such alterations play a role in the development of the disease. The objectives of this thesis were: 1) to use microsatellite analysis to examine DNA extracted from severe dysplasia, carcinoma *in situ* (*CIS*) and SCC for novel alterations on 11q; 2) to determine at what stage of oral cancer development the alteration occurred by performing microsatellite analysis on a spectrum of stages of oral premalignant lesions (hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, *CIS*) as well as invasive SCC. The data obtained was compared to 2 previously studied hotspots on 11q: the *int2* (11q13) and *D11S1778* (11q22-23); and 3) to determine the significance of allelic imbalance (AI) at *int2* (11q13) and *D11S1778* (11q22-23) to the progression of

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oral premalignant lesions by comparing frequencies of loss for different locus in low-grade dysplasia with known outcome, i.e. low-grade lesions that did not progress into cancer with morphologically similar lesions that did develop into SCC.

In summary, the data suggest that at least 3 regions of alteration are present on 11q in both oral premalignant and malignant lesions and that 1 of these regions, identified as containing the novel marker *D11S4207*, might play a significant role in the early development of the disease. The data further support the use of these markers to identify progression risk for early oral premalignant lesions.

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LIST OF ABBREVIATIONS

AI	allelic imbalance
ATM	ataxia telangiectasia mutated
BAC	bacterial artificial chromosome
BFB	Breakage-fusion-bridge
CCND1	cyclin D1
CIS	carcinoma *in situ*
dmin	double-minute chromosomes
DNA	deoxyribonucleic acid
FGF	fibroblast growth factor
FISH	fluorescence *in situ* hybridization
H & E	hematoxylin and eosin
hsrs	homogeneously staining regions
HNSCC	head and neck squamous cell carcinoma
HST1	heparin secretory transforming factor 1
LOH	loss of heterozygosity
Mb	megabase pair
MEN1	multiple endocrine neoplasia type 1
OPL	oral premalignant lesion
PCR	polymerase chain reaction
SCC	squamous cell carcinoma

TSG   tumor suppressor gene

WHO   World Health Organization

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DEDICATION

To My Mom and My Dad

Who Love Me and Whom I Love Forever

I.   INTRODUCTION

I.1.   Overview

Oral cancer is the sixth most common cancer in the world, accounting for about 3% of all

new cancers in Western countries (Harras *et al.*, 1996; Greenlee *et al.*, 2001) and up to

40% of all cancers in places such as India (Saranath *et al.*, 1993). Despite refinement of

surgical techniques and adjuvant therapies, the prognosis of oral cancer has remained

unchanged for decades with 5-year survival rates of 40-50% in the Western world and

even lower in undeveloped countries (20-43%) (Rao and Krishnamurthy, 1998; Greenlee

et al., 2001). The major cause of this high mortality rate is the late-stage at which most

cancers are identified, resulting in a high local recurrence and formation of second

primary malignancies even in successfully treated cases (Cooper *et al.*, 1989; Day and

Blot, 1992; Lippman and Hong, 1989; Shikhani *et al.*, 1986). The development of novel

approaches for the prevention, early detection, and effective treatment of this disease are

critical to improving outcome. Such approaches are dependent on a better understanding

of the molecular and cellular mechanisms underlying the tumorigenesis process.

Molecular analyses of oral cavity tumors have uncovered a number of recurrent genetic

events that appear to underlie the development of the disease. Among these changes are

frequent alterations to loci (and genes) on chromosome 11q. Microsatellite analysis of

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oral tumors suggests the presence of at least 2 regions on this chromosomal arm containing putative TSGs or oncogenes. These regions include 11q13 and 11q22-23, each of which contain loci that show frequent allelic imbalance in tumors, with such alteration occurring in 30-80% of cases (Dwight et al., 2000; Ah-See et al., 1994; Venugopalan et al., 1998; D'Adda et al., 1999; Uzawa et al., 1996; Lazar et al., 1998; Hui et al., 1996). The region around 11q13 is of particular interest in that amplification of this region (as identified with fluorescent in situ hybridization or FISH) has been reported in 30-50% of oral cancers (Fujii et al., 2001; Ott et al., 2002; Bayerllein et al., 2000; Alavi et al., 1999; Williams et al., 1993; Muller et al., 1997; Meredith et al., 1995; Fortin et al., 1997). Several genes potentially important for creating the malignant phenotype are located at 11q13 or 11q22-23 including *int2, FGF4, EMS1, RIN1, MEN1, PAK1, ATM* and the most commonly reported gene, *cyclin D1*. However, the evidence in support of the involvement of the majority of these genes is still limited.

There is little information on the involvement of alterations on 11q in oral premalignant lesions. Such alterations have been reported as being present in head and neck premalignant lesions (El-Naggar et al., 1995; Califano et al., 1996; Poh et al., 2001). However, the numbers of cases used in these studies is small and the association with the degree of dysplasia is reported in only one of these studies (Poh et al., 2001). That study suggested that 11q alterations may be occurring late in carcinogenesis, between severe dysplasia and SCC (Poh et al., 2001). There is also some preliminary evidence from this laboratory that suggests that allelic imbalance at 11q is associated with an increased risk for progression (Rosin et al., 2000). These studies need to be confirmed using a larger

number of cases and expanding the analysis to other loci on this arm in order to better

identify both the sites containing genes of importance to progression of oral premalignant

lesions and to determine whether or not such markers can be used as indicators of risk of

progression for early lesions.

The goal of this thesis was to examine oral cancers and premalignant lesions for

alteration to markers on the 11q arm in order to better define the timing and frequency of

alterations at 11q. A second goal was to determine whether there was an association of

alteration at 11q with clinical information and prognosis. A final objective was to use

microsatellite analysis to begin to define novel regions of alteration in oral premalignant

lesions that could later be studied for candidate tumor genes located at 11q.

The following introduction begins with a summary of the clinical and histological

alterations that occur during oral carcinogenesis and the limitations of histopathological

criteria in identifying outcome for lesions with no dysplasia or with low-grade dysplasia.

A brief summary of genetic alterations associated with oral tumorigenesis is then given

followed by a more specific presentation of 11q alterations and cancer, with an emphasis

on oral squamous cell carcinomas and premalignant lesions.

I.2. Clinical, histological and molecular alterations during oral carcinogenesis

I.2.1. Clinical alterations during oral carcinogenesis

A premalignant lesion, as defined by the World Health Organization (WHO), is 'morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart' (WHO, 1978). This definition was reaffirmed in 1994 by the International Collaborative Group on Oral White Lesions (Axell et al., 1996).

In the oral cavity, premalignant lesions most frequently present as leukoplakia and erythroplakia (Axell et al, 1996). Leukoplakia (Figure 1, A) means "white patch". It occurs on membranes such as the mucosa of the oropharynx, larynx, esophagus, and genital tract. The term leukoplakia should not be applied to all the white patches of these mucosal surfaces. 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).

Likewise, the WHO defines erythroplakia (Figure 1, B) as a fiery red patch that cannot

be characterized clinically as any other definable lesion (Pindborg *et al.*, WHO, 1997). In contrast to leukoplakia which are often benign in nature, lesions with erythroplakia are generally either already malignant or are at high-risk for transformation to malignancy (Bouguot and Ephros, 1995; Mashberg, 1977; Waldron and Shafer, 1975).

It is generally believed that a premalignant lesion that most frequently presents clinically as leukoplakia or erythroplakia often precedes oral cancer. However, the majority of leukoplakias will not progress to cancer.

Figure 1.   Clinical presentation of oral premalignant lesions

A   B
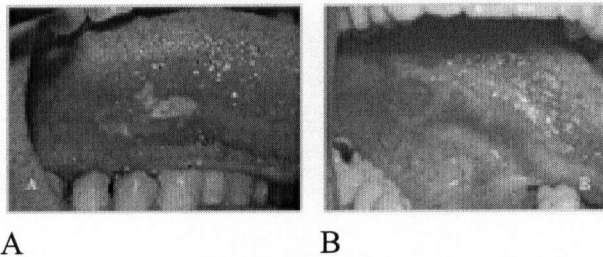
A: Leukoplakia on right latero-ventral tongue; B: Erythroplakia on right latero-ventral tongue.

A number of clinical factors have been found to affect the malignant risk of oral premalignant lesions. These include the appearance, size, site and duration of the lesion, the consumption of tobacco and alcohol, and history of head and neck cancer, or of *candida* infection (van der Waal *et al.*, 1997). The following is a brief discussion of these attributes.

Leukoplakia can be classified as either homogeneous or non-homogeneous, according to

the clinical appearance of oral premalignant lesions. Homogeneous leukoplakias are those lesions showing a consistent color and texture; these lesions have a lower risk for malignant transformation compared to non-homogeneous leukoplakia (Axell *et al.*, 1996; Pindborg *et al.*, WHO, 1997).

Leukoplakia occurs throughout the oral cavity, with those in the buccal and mandibular sites being the most common. Leukoplakia located on the floor of the mouth, ventrolateral surface of the tongue and soft palate have an increased cancer risk (Schell and Schonberger, 1987; Mashberg and Meyers, 1976). 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).

Clinical risk factors help clinicians decide whether an oral lesion should be biopsied for histopathological evaluation and also help in the overall judgment of malignant risk of the lesions. However, these clinical factors have a limited ability to precisely predict cancer risk. Currently, histological criteria represent the gold standard for judging the risk of malignant transformation for oral premalignant lesions.

I.2.2. Histological alterations during oral carcinogenesis

I.2.2.a.   *Normal oral mucosa*

The oral cavity is lined by oral mucosa, which consists of stratified squamous epithelium and a layer of connective tissue called the lamina propria. The stratified squamous epithelium can be divided largely into basal and prickle cells. The lamina propria lies underneath the epithelium and contains blood vessels and lymphatic vessels, small nerves, fibroblasts, collagen and elastic fibers. It functions to nourish and support the epithelium. At the boundary of the epithelium and connective tissue is a layer of basal cells. Some of these basal cells are "stem cells" of the tissue which possess the ability to replicate themselves to replace cells lost, during tissue turnover. When a basal "stem" 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 where they shrink in size, become long and flat, and are eventually desquamated. Oral epithelium is usually non-keratinized except for mucosa lining the attached gingiva, hard palate, dorsal surface of the tongue, and lips. The majority of oral premalignant and malignant lesions arise from this stratified squamous epithelium of oral mucosa and the malignant tumors are called squamous cell carcinoma (SCC).

I.2.2.b. Oral premalignant lesions and squamous cell carcinoma (SCC)

Oral SCC is believed to evolve from normal tissue that develops premalignant lesions with these lesions increasing in severity and eventually becoming malignant. To assess the risk of malignant transformation of leukoplakia or erythroplakia, a biopsy is taken of the clinical lesion and examined for the presence and degree of dysplasia. The World Health Organization has established the following criteria for histological diagnosis of oral dysplasia (1978):

1. Loss of polarity of the basal cells

2. The presence of more than one layer having a basaloid appearance

3. Increased nuclear/cytoplasm ratio

4. Drop-shaped rete-ridges

5. Irregular epithelial stratification

6. Increased numbers and abnormality of mitotic figures

7. The presence of mitotic figures in the superficial half of the epithelium

8. Cellular pleomorphism (variation in shape and size)

9. Nuclear hyperchromatism (dark staining nuclei)

10. Enlarged nucleoli

11. Loss of intercellular adherence

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

Architecturally, dysplastic lesions are further divided into mild, moderate, and severe forms depending upon how much of the tissue is dysplastic. Mild dysplasia is a lesion in which the dysplastic cells are confined to the lower one third of the epithelium. Moderate dysplasia is a lesion in which the dysplastic cells are evident in about half the thickness of the epithelium. Severe dysplasia is a lesion in which the dysplastic cells have filled the lower two-thirds of the epithelial thickness. In carcinoma *in situ* (*CIS*), the dysplastic cells occupy the entire thickness of the epithelium (bottom to top changes) although the basement membrane is still intact (Lumerman *et al.*, 1995). Invasion of dysplastic cells through the basement membrane into the underlying stroma and/or the dissemination of these cells to other sites through lymphoid and circulatory systems are events associated with development of invasive SCC.

A histological progression model has been established for oral cancer (Figure 2). In this model, oral cancers progress through hyperplasia and increasing degree of dysplasia (mild, moderate and severe) to *CIS*, and finally break through the basement membrane and become SCCs. Severe dysplasia and *CIS* usually are grouped together as high-grade dysplasia, because both 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).

The presence and the degree of dysplasia are believed to have a huge impact on the malignant risk of the premalignant lesions. All studies to date have shown that leukoplakia with dysplasia have higher malignant risk than those without dysplasia

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(Waldron and Shafer, 1975; Lumerman *et al.*, 1995). A large clinical study by Silverman *et al.* (1984) found that during a mean follow-up period of 7.2 years, more than 36% of leukoplakia lesions with epithelial dysplastic features eventually underwent malignant transformation, whereas those leukoplakia without dysplasia only demonstrated a malignant rate of 15%. The relationship of the malignant risk and degree of dysplasia is further demonstrated by parallel studies from 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). As a result, the gold standard for judging the malignant potential of premalignant lesions in these organs and systems, including the oral cavity, is the presence and degree of dysplasia.

It is important to note, however, that there are limitations in the use of histological criteria to predict malignant risk of oral premalignant lesions. These criteria have a good predictive value for high-grade dysplasia, severe dysplasia and *CIS*, which have a high chance of progressing into invasive lesions (Banoczy and Csiba, 1976; Schepman *et al.*, 1998). However, histology is a poorer predictor of malignant risk for low-grade dysplasia (mild and moderate dysplasia). Low-grade lesions that ultimately progress to tumors appear histologically mimic to those that regress or remain unchanged over extended period of time. Since such lesions represent the majority of premalignant lesions, a way to differentiate progressing from non-progressing would have a large impact on prevention of oral cancer. Advances in molecular techniques may provide a new direction to solving this problem.

Figure 2. Histological progression model for oral premalignant and malignant lesions
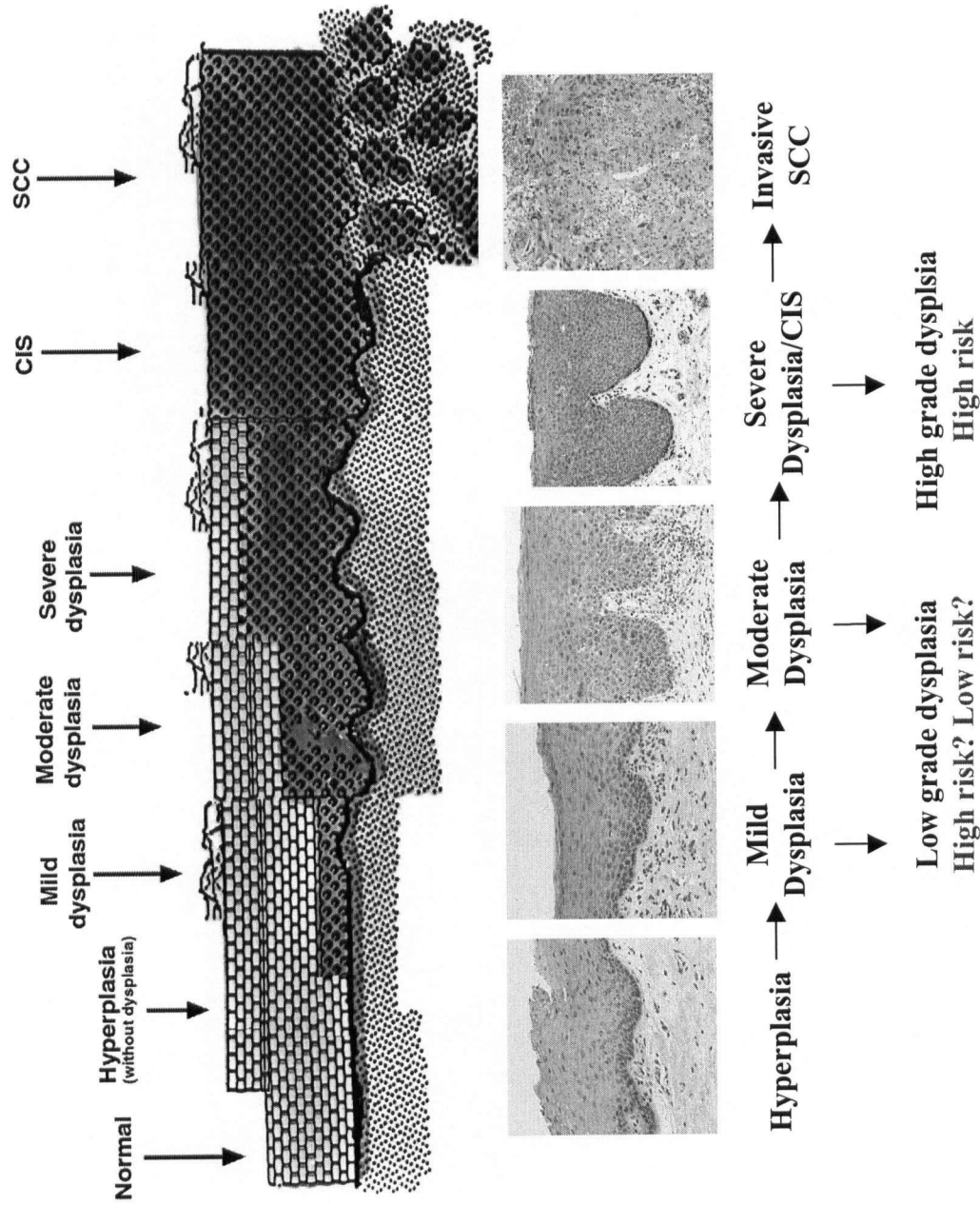
Histological progression from hyperplasia, increasing degree of dysplasia (mild, moderate, severe dysplasia), *CIS* to invasive SCC. The top panels show the cartoon of histological progression model. The lower panels show photomicrographs of histological progression (H & E, x40, original magnification).

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I.2.3. Molecular alterations during oral carcinogenesis

I.2.3.a. The importance of studying genetic changes in cancer progression

It is now widely accept that cancer develops through a series of genetic events that parallel the histopathological progression of a lesion through premalignant stages to carcinoma *in situ*, and, finally, into an invasive lesion. Molecular progression models outlining these genetic changes were established in colon cancer first (Vogelstein *et al.*, 1988), and then developed for many other solid tumors. According to these models, different genetic changes occur in different stages of the disease process and play an integral role in the progression of the lesion to cancer. Many of these genetic events take place well before a given tumor produces clinical symptoms and often before a benign lesion or focus of dysplasia develops into an invasive cancer (Sidransky, 1997). This suggests that if such changes could be used to distinguish lesions with an elevated risk of developing into cancer.

As an example of such an approach, there is substantial evidence that microsatellite analysis can be used to predict outcome for oral premalignant lesions (Califano *et al.*, 1996; Partridge *et al.*, 1998; 1999). In a recent report from this laboratory, it was shown that loss of heterozygosity (LOH) at 3p and/or 9p was one of the earliest changes associated with an increased risk of malignant transformation (Rosin *et al.*, 2000). Low-grade dysplasia with LOH at 3p and/or 9p had approximately 4 times the risk of

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developing into cancer compared to those without such loss. Cases that had LOH at 3p and/or 9p with additional loss on other chromosome arms (4q, 8p, 11q, or 17p) had a 34-fold increase in relative cancer risk. These results are supported by a number of studies from other laboratories (Partridge *et al.*, 1998; 1999; Lippman and Hong, 2001) and strongly support the use of molecular markers to predict cancer risk of premalignant lesions. This article will be discussed in more detail in section I.2.3.f.

Genetic alterations have also been found to correlate with clinical parameters of cancer, such as recurrence, metastasis and poor prognosis. This has been observed for many types of cancer. For example, LOH of loci at 3p25.1, 13q12 or 17p13.3 have been associated with lymph node metastasis and poor prognosis of breast cancers (Hirano *et al.*, 2001). Deletion at 3p25.3-p23 has been shown to be associated with metastasis of endocrine pancreatic carcinoma (Barghorn *et al.*, 2001). A putative tumor suppressor gene (TSG) located at 20p11.23-p12 has been reported to be involved in the development of prostate cancer metastases (Goodarzi *et al.*, 2001). LOH at 5q21-22 has been reported to be linked to known oral SCC etiologic factor (human papilloma virus) and the prognosis of patients (Mao *et al.*, 1998). Another study has concluded that microsatellite analysis for 18q21.1 and 9p21-22 is capable of predicting the clinical outcome of bladder cancer patients (Uchida *et al.*, 2000). All these papers have shown that genetic alterations can be used to predict clinical outcomes and help to decide treatment.

Another reason for studying genetic alterations is to facilitate the development of new regimen such as treatment of gene therapy for premalignant lesion and cancers. The

efforts in cancer gene therapy focus on four types of approaches: chemogene therapy, such as the introduction of genes that confer susceptibility to chemotherapeutics; immunogene therapy, which involves modulation of the patient's immune response capacity; tumor suppressor gene-mediated inhibition of tumor growth promoters like oncogenes and cytokines; and inhibitors of tumor angiogenesis and invasiveness (Vogelstein and Kinzler, 1998). Each of these approaches requires a basic understanding of the genetic background of a patient's lesion. For example, in gene therapy, the transfer of TSGs to cancer cells to suppress tumorigenesis requires the identification of such genes in a patient's lesion.

A final example of how studies of genes altered in tumorigenesis can be used to tailor intervention and treatment strategies is exemplified by carriers of *APC* (adenomatous polyposis coli) mutations. Such individuals have a higher risk of developing colon cancer (Nagase *et al.*, 1992; Miyoshi *et al.*, 1992; Powell *et al.*, 1992; Smith *et al.*, 1993). In such situations, a prophylactic colorectomy is used to reduce the incidence of this disease dramatically (Tomlinson *et al.*, 1997).

I.2.3.b.   Oncogenes and tumor suppressor genes (TSGs)

Nowell proposed in 1976 that neoplastic transformation occurred in a single cell that had a critical genetic alteration that gives it a growth advantage over its neighboring cells (Nowell, 1976). This was followed by successive rounds of mutation and expression

with the accumulation of multiple genetic alterations during the progression of the disease. In the head and neck region, 7-10 independent genetic events are believed to be involved in the production of invasive SCC (Renan, 1993). This theory has been supported by a genetic progression model for head and neck cancer developing by Califano *et al.* (1996). Based on this model, specific genetic alterations occur during the progression from normal mucosa through increasing degrees of dysplasia and finally to invasive SCC. These genetic events include the mutation of a number of critical genes that control the processes of cellular proliferation and differentiation in a tissue. The genes associated with carcinogenesis are classified into two main groups: oncogenes and tumor suppressor genes (TSGs).

Oncogenes, which originally were identified as the transforming genes in viruses, are altered forms of normal cellular genes called proto-oncogenes. In human cancers, proto-oncogenes are frequently located adjacent to chromosomal breakpoints and are targets for mutation. The products of proto-oncogenes play a key role in regulating the cascade of events that maintains the ordered processes through the cell cycle, cell division, and differentiation. More than 50 different proto-oncogenes have been identified, coding for proteins that function as growth factors, growth factor receptors, cytoplasmic second messengers, protein kinases, nuclear phosphoproteins, transcription factors and others. They can be roughly subdivided into two groups. One class of genes rescues cells from senescence and programmed cell death, acting as immortalizing genes. A second class of genes reduces growth factor requirements and induces changes in cell shape that results in a continuous proliferative response (Vogelstein and Kinzler, 1998).

A number of mechanisms have been described for the mutation of these proto-oncogenes to oncogene (called activation) including point mutations, gene amplification and chromosome translocations (Vogelstein and Kinzler, 1998). These alterations can involve the coding region of the gene, resulting in alteration of structure and activity of coded proteins. Alternatively they can alter the regulating region of a gene, resulting in the inappropriate expression of the gene. This resulting genetic alteration is autosomal dominant, meaning that only one of the two gene copies needs to be changed for an effect to be observed.

The mechanism of gene amplification requires further comment at this time, since the region to be studied in this thesis (11q13) has often been reported as being amplified during carcinogenesis. The term gene amplification refers to an increase in copy number of a gene or a specific, subchromosomal region. Gene amplification almost always results in the overexpression of one or more genes contained on the amplicon. The amplification and consequent overexpression of a number of genes can act to confer a selective advantage on a cancer cell.

In contrast to oncogenes, tumor suppressor genes (TSGs) are a group of genes encoding proteins, which, through a variety of mechanisms, function to negatively regulate cell growth and differentiation pathways. The functions of TSGs must be lost in order for tumorigenesis to occur. Loss of a TSG's function requires the inactivation of both gene copies (maternal and paternal) through mutation, deletion, or other mechanisms such as methylation (Knudson *et al.*, 1977, 1985, 1993). The most commonly reported process of

TSG loss in sporadic tumors involves the inactivation of a gene by a point mutation followed by inactivation of the second copy by any one of many mechanisms including deletion. A commonly used procedure to identify such deletion is the microsatellite assay, to be discussed in a later section. This assay is the primary techniques used in this thesis.

Many tumor suppressor genes have been localized and identified, including *p53, RB* (retinoblastoma), *VHL* (the gene responsible for von Hippel-Lindau syndrome), *FHIT* (fragile histidine trial), *p16, DPC4, APC* (adenomatous polyposis coli), *doc-1* (deleted in oral cancer), *TSC2, BRCA1, NF-1, NF-2 and WT-1* (Mao *et al.*, 1996; Reed *et al.*, 1996; Gleich *et al.*, 1996; Todd *et al.*, 1995; Largey *et al.*, 1994; Pavelic *et al.*, 1997; Uzawa *et al.*, 1994; Mao *et al.*, 1996; and Kim *et al.*, 1996; Latif *et al.*, 1993; Kanno *et al.*, 1994; Sparks *et al.*, 1998). Although the cellular functions of tumor suppressor proteins, such as p105-RB, p53 and p16, are becoming increasingly well understood, others remain largely undefined. It is clear, however, that the tumor suppressor proteins will exhibit a variety of functions within the cell.

Functional loss of TSGs is one of the most common genetic alterations during carcinogenesis including that of the head and neck region. Thus, the defining of chromosomal regions that harbor biologically important suppressor genes may have broad practical implications not only on tumor progression, but also on the clinical management of cancers and premalignant lesions.

I.2.3.c. Oncogenes and TSGs in oral premalignant and malignant lesions

Few oncogenes have been identified as showing mutation in head and neck or oral squamous cell carcinoma, although changes in the expression of many potential oncogenes has been reported. These genes include *ras, cyclin-D1, myc, erbB, bcl-1, int2, CK8* and *CK19* (Kiaris *et al.,* 1995; Lese *et al.,* 1995; Saranath *et al.,* 1993; Warnakulasuriya *et al.,* 1992; Wong *et al.,* 1993; Bartkova *et al.,* 1995; Xu *et al.,* 1995; Masuda *et al.,* 1996; Riviere *et al.,* 1990). 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* have been 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 to a dysregulation of the expression of this gene resulting from a downstream effect of another mutation.

On the other hand, many studies have focused on the role of TSGs in oral carcinogenesis. Some of the TSGs involved in head and neck cancers include *p53, Rb (retinoblastoma)*, and *p16INK4A* (Gallo *et al.*, 1999; Gleich *et al.*, 1996; Jares *et al.*, 1999; Liggett *et al.*, 1996; Papadimitrakopoulou *et al.*, 1997; Partridge *et al.*, 1998 and 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-Lindau 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 molecular analysis techniques has rapidly revolutionized the ability to look at these genetic alterations. Most studies on TSGs, particularly those in oral premalignant lesions, use microsatellite analysis to identify loss of heterozygosity (LOH) in DNA extracted from epithelial cells belonging to these lesions. This is also the major technique used to conduct research for this thesis.

I.2.3.d.   *Microsatellite analysis amd loss of heterozygosity (LOH) studies*

Microsatellite analysis is a powerful molecular technique for identifying and studying TSGs. It can detect changes from as small as a few thousand nucleotides to as large as a whole chromosome in one of a pair of chromosomes. The assay is designed to assess

alterations to polymorphic chromosomal regions that map close to or within putative or known TSGs.

Two methods are available for the study of LOH: restriction fragment length polymorphism (RFLP) analysis and microsatellite analysis. The advantages of using microsatellite analysis are twofold. 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 [γ-p^{32}] end-labeled PCR-based approach is much more sensitive than RFLP analysis and requiring only a small amount of DNA (5 nanograms or less per reaction). This aspect is critical for the study of premalignant lesions given the small size of such lesions. Another advantage is that microsatellite makers can be used on DNA extracted from paraffin-embedded archival samples in addition to fresh or frozen samples. This allows one to use the large number of specimens in hospital archives to perform retrospective analysis of lesional progression. The following is a brief description of this procedure.

Microsatellites contain runs of short and tandemly repeated sequences of di-, tri-, or tetra-nucleotides, such as –GTGTGT-, -GTAGTAGTA-, or –GTACGTACGTA- (Figure 3). The number of such tandem repeats is highly polymorphic in the population. Thus, individuals often contain a different number of copies of the repeat in maternal and paternal alleles. These regions are well interspersed throughout the human genome (e.g., estimated every 30-60 kilobase pairs (kb) for CA repeats) and are highly conserved

through successive generations (Ah-See *et al.*, 1994; Beckman and Weber, 1992).

The assay involves amplification of region containing microsatellites with polymerase chain reactions (PCR) using radioactively labeled nucleotides. The reaction products are separated on polyacrylamide gel, with separate bands being produced whenever on individual is heterozygous for a region (i.e., when paternal and maternal copies of the region contain different numbers of repeats). The intensity of the two alleles is compared for DNA isolated from normal and tumor or dysplasia cells in a tissue. An alteration in intensity of the bands in the lesion DNA compared to normal DNA is scored as LOH (see section V.13. for details).

The observation of a high frequency of LOH in a microsatellite region in a set of tumors or dysplasias is suggestive of the presence of a putative suppressor gene nearby. However, it is important to note that microsatellite analysis cannot distinguish between duplication or low-level amplification of an allele and deletion of an allele, particularly if there are contaminating normal cells within the tumor (Ah-see *et al.*, 1994). Both alterations would result in a change in the relative signal intensities for the two alleles in the lesion DNA. To determine whether a deletion or amplification has occurred, other assays are used. One approach involves the use of fluorescence *in situ* hybridization (FISH) on cell or tissue preparations to determine the number copies of the region in cells. Alternatively, Southern blots could be used with specific gene probes. Both procedures are time-consuming. The latter procedure requires significantly more DNA than the microsatellite analysis. Other procedures involve sequencing the gene to

determine whether a mutation exists. This process also requires large quantities of DNA or RNA.

Because of the difficulty in differentiating amplification from deletion as a source of alteration to microsatellite signals, many investigators use the term allelic imbalance (AI) to describe the change of signal intensity observed with microsatellite analysis. I will use both terms indiscriminately throughout this thesis.

Figure 3. Schematic illustration of microsatellite analysis and AI.

Normal cells   Abnormal cells
   (N)   (A)
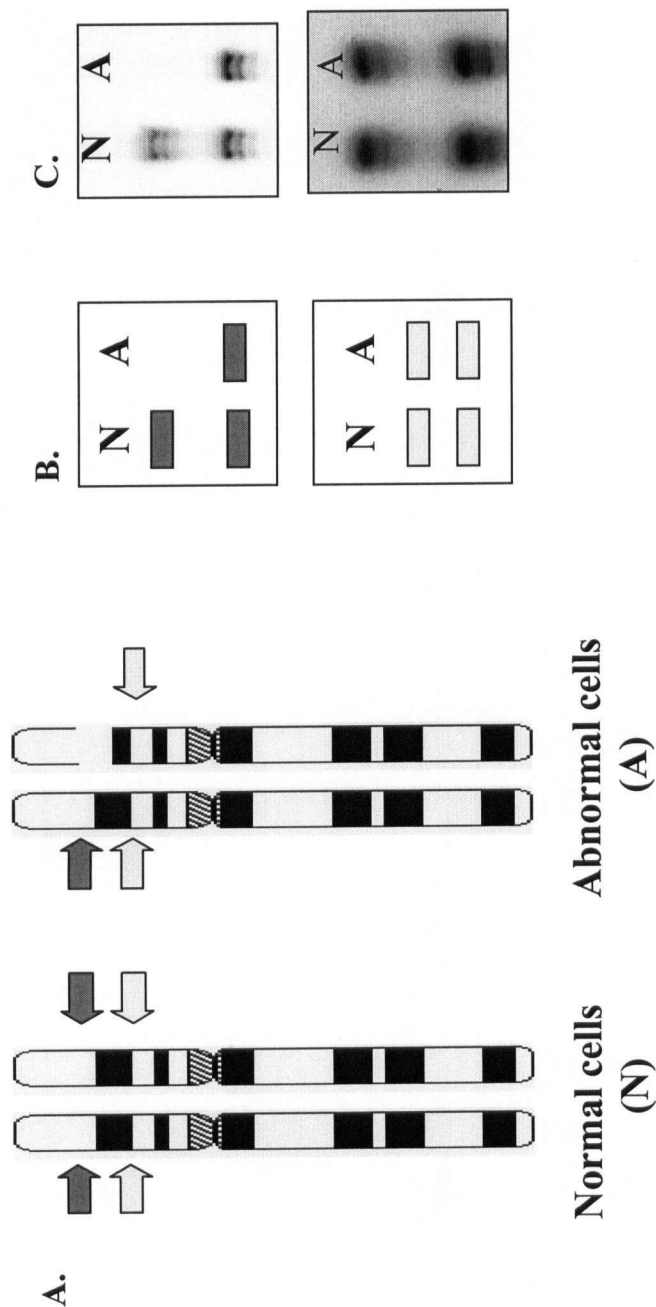
A. Schematic representation of a single chromosme pair from a normal specimen and an abnormal specimen containing a loss of a chromosomal band. Green arrows indicate region of retention. Red arrows indicate region of loss.

B. Schematic of PCR amplified fragments as they are visualized on a denaturing polyacrylamide gel. Red fragments show both alleles retained in normal cells and one allele lost in abnormal cells. Green fragments show both alleles retained in both tissue types.

C. Actual gel showing same LOH results as B.

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I.2.3.e.   Microsatellite analysis of oral cancer and premalignant lesions

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; Ogawara *et al.*, 1998; Mao *et al.*, 1998;

Miyakawa *et al.*, 1998; Nakanishi *et al.*, 1999; Partridge *et al.*, 1999; Yamamoto *et al.*,

1999). Such chromosomal regions include: 3p, 4q, 5q, 8p, 9p, 10q, 11q, 13q, 14q, 17p,

18q and 22q (Califano *et al.*, 1996; el-Naggar *et al.*, 1995; Pershouse *et al.*, 1997; Shah *et

al.*, 2000; Uzawa *et al.*, 1994; Resto *et al.*, 2000; Field *et al.*, 1995; Partridge *et al.*, 1994,

1996, 1999; Okami *et al.*, 1998; Lee *et al.*, 1997; Papadimitrakopoulou *et al.*, 1998; Mao

1998; Ishwad *et al.*, 1999; Miyakawa *et al.*, 1998).

In contrast, there are fewer studies of genetic changes in oral premalignant lesions. This

is partly due to the greater difficulty in accessing premalignant lesions as compared to

oral SCC in the larger hospitals. In addition, oral premalignant lesions are generally

much smaller than SCC, and yield smaller quantities of DNA. Most of the studies on oral

premalignant lesions are limited in either the number of samples used and/or the number

of regions assayed. In addition, often the degree of dysplasia is not mentioned. The

following is a discussion of a few critical articles that have been keynote to the study of

progression of oral premalignant lesions.

I.2.3.f. Genetic progression model for head and neck cancer

In a hallmark study by Califano *et al.* (1996), LOH patterns were examined at 10 loci in a wide spectrum of lesions of the head and neck region, including hyperplasia, dysplasia, *CIS* and SCC in order to determine whether there was an association of such alteration with the histological progression of the disease. The study showed that LOH occurred early in disease development. Loss of at least one locus occurred in nearly all samples of dysplasia and *CIS* but was present in one third of hyperplasias. Although the authors suggested that it was the accumulation and not necessarily the order of genetic events that determined progression, they also suggested that some losses were more likely to occur early in the development of the disease oral cancer. Others are at later stages. A molecular progression model was proposed with LOH at 9p an early event associated with the transition from normal to benign hyperplasia. LOH at 3p and 17p were associated with development of dysplasia, while *CIS* and SCC were characterized by additional loss at 4q, 6p, 8, 11q, 13q and 14q.

A major drawback of the study is that all the dysplasias were grouped together. It is well accepted that with increasing degrees of dysplasia there is an increasing risk of malignant transformation. While the majority of mild dysplasia will not progress into cancer, severe dysplasia, similar to *CIS*, has a much higher probability of cancer progression. Identification of genetic profiles in early dysplasia and late dysplasia is therefore critical

to understanding progression in the disease.

Our research lab has further refined this molecular progression model for oral SCC by investigating all degrees of oral dysplasias using multiple primers on 8 chromosome arms (Rosin *et al.*, 2000; Poh *et al.*, 2001; manuscript in preparation) (see Table 1, Figure 4).

Table 1.   **AI in oral lesions**

Chromosome region	Hyperplasia	Degree of dysplasia			SCC
		Mild	Moderate	Severe	
3p14	4/31 (13)[a]	10/29 (34)	11/24 (46)	10/23 (43)	28/34 (82)
4q26-31	0/31 (0)	2/28 (7)	2/20 (10)	8/21 (38)	13/33 (39)
8p21-23	0/32 (0)	2/28 (7)	6/23 (26)	5/23 (22)	17/34 (50)
9p21	2/32 (6)	11/31 (35)	14/24 (58)	18/23 (78)	26/34 (76)
11q13-22	1/32 (3)	4/30 (13)	2/23 (9)	2/23 (9)	18/34 (53)
13q12-14	1/33 (3)	1/30 (3)	1/24 (4)	4/21 (19)	15/33 (45)
14q31-32	0/33 (0)	7/28 (25)	10/23 (43)	7/23 (30)	12/33 (36)
17p11-13	0/34 (0)	4/30 (13)	8/24 (33)	16/23 (70)	21/33 (64)

[a] Values given as number of samples showing loss/total number of informative cases. Values in parentheses are percentages.

The data support the findings of the Califano study that an accumulation of genetic alterations is critical for tumor progression, and that there are preferred patterns of allelic loss associated with different degrees of dysplasia. These patterns include the following:

Loss associated with lesions without dysplasia or low-grade dysplasia: As shown in Table 1, the most common change in hyperplasia occurred at loci on 3p (13% of hyperplasia). The most frequent loss in mild dysplasia occurred at 3p, 9p, and 14q. Frequencies of loss at 9p and 14q are significantly higher in mild dysplasia compared to hyperplastic lesions ($P < 0.01$). Loss at 3p approached significance ($p = 0.07$, probably will be significant with larger sample number).

Loss associated with high-grade lesions: LOH at 17p and 4q associated with transitions between moderate and severe dysplasias/*CIS*. LOH in severe dysplasia/*CIS* are significantly higher than in moderate dysplasia at 17p ($p = 0.02$) and at 4q ($p = 0.07$, approaching significance).

Loss associated with invasion: Frequencies of Loss at 4 loci increase significantly between severe dysplasia/*CIS* and SCC: 3p ($p = 0.0038$), 8p ($p = 0.05$), 11q ($p < 0.001$), and 13q ($p = 0.04$). These data suggest the possibility that genes in these regions play a significant role with late stage events such as invasion and metastasis.

Figure 4. Molecular progression model of oral cancer proposed by Rosin and Zhang (2001).
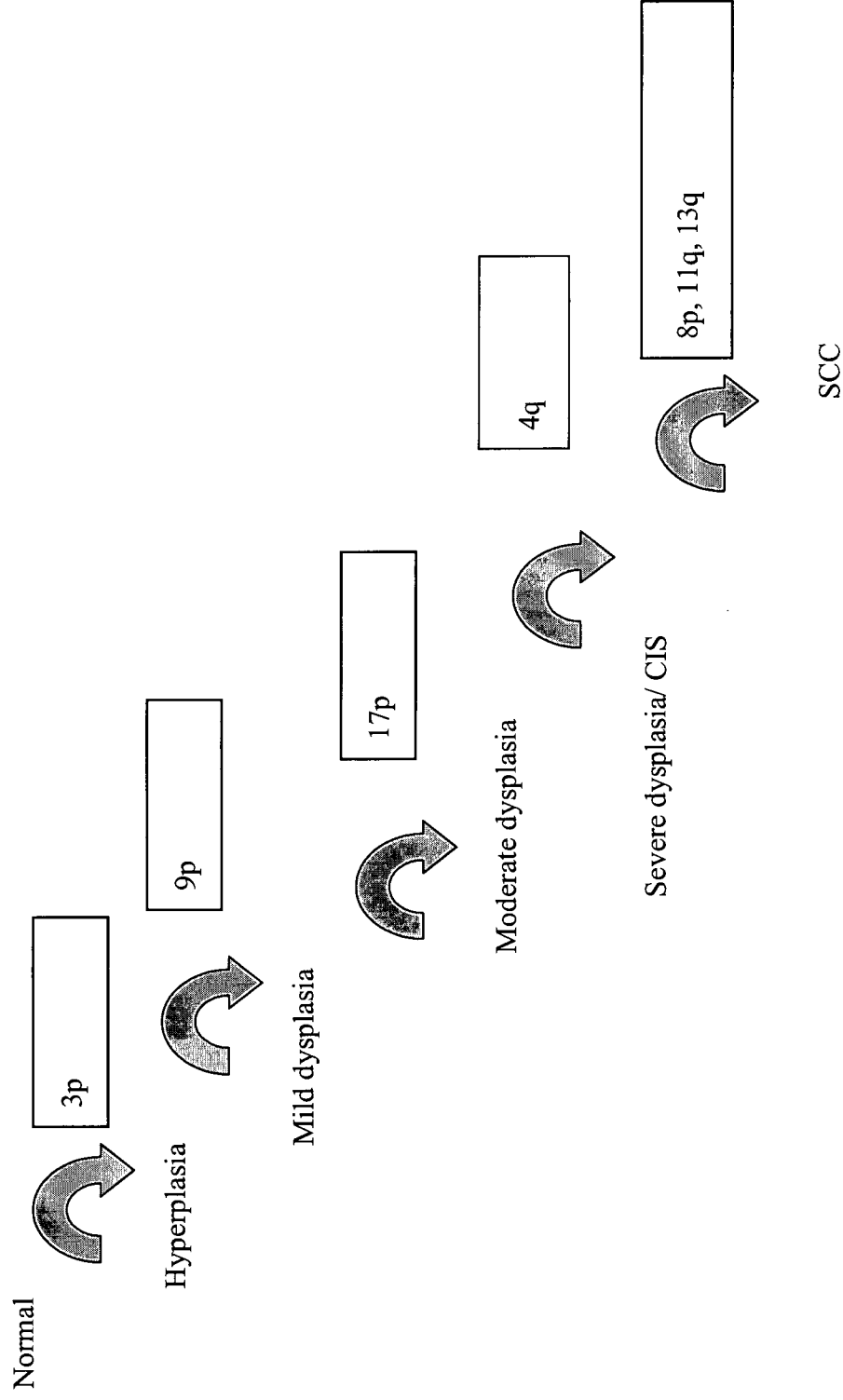
Oral cancer is a multistep process with accumulation of genetic changes, which are parallel to the histological lesions.

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I.2.3.g. Prediction of risk of progression for oral premalignant lesions (OPL)

The aforementioned studies suggest that LOH is a common event in OPLs and can occur early in carcinogenesis. A few reports support the use of this assay to improve our ability to predict risk of malignant transformation for oral premalignant lesions.

In 1996, Mao et al. (Mao et al., 1996) examined 84 oral leukoplakia samples from 37 patients enrolled in a chemoprevention study. The samples were analyzed for LOH at 9p21 and 3p14. LOH at either or both loci was observed in 19 of these patients and this loss was strongly associated with cancer progression. Seven (37%) of the 19 positive cases later developed SCC. In contrast, only 1 of 18 cases (6%) without LOH progressed to cancer.

Partridge and co-workers also looked for an association between LOH pattern and progression. In an early study in that laboratory, they reported an association between multiple LOH in oral premalignant lesions and increased progression risk (Partridge et al., 1998). This result was again observed in a more recent study from that laboratory (Partridge et al., 2001) involving 78 oral premalignant lesions, histologically diagnosed with hyperplasia or dysplasia, all from patients with no prior history of oral cancer. In half of the patients, the lesions progressed, usually at or adjacent to the original site. Progressing lesions were characterized by the presence of multiple regions of LOH, with loss of 9p or 3p present in 94% of lesions.

As mentioned previously in section *1.2.3.f.*, we recently have completed a retrospective study that restricted the focus to lesions without – or with minimal – dysplasia (Rosin *et al.*, 2000). These are the lesions that are the most difficult for clinicians to manage. One hundred and sixteen biopsies of oral premalignant lesions were examined to see if a correlation existed between LOH at 7 chromosome arms (3p, 4q, 8p, 9p, 11q, 13q, and 17p) and progression risk. None of the patients had a history of cancer prior to the studied hyperplasia or mild/moderate dysplasia. Twenty-nine of the 116 OPLs progressed to cancer. The progressing lesions showed not only a significantly higher number of LOHs, but also characteristic LOH patterns (Figure 4). LOH at 3p &/or 9p was present in 97% of progressing lesions, suggesting that loss at these arms may be a progression prerequisite. However, since many non-progressing lesions also showed losses at 3p and/or 9p, such loss alone is probably insufficient for malignant transformation. Indeed, cases with LOH at these arms but no others showed only a 3.8-fold increase in relative risk for cancer development. In contrast, individuals with additional losses at 4q, 8p, 11q, 13q, or 17p showed a 33-fold increase in relative cancer risk. In non-progressing cases, additional losses were uncommon. These results suggest that LOH patterns may differentiate 3 progression risk groups: Low, with retention of 3p and 9p; intermediate, with losses at 3p and/or 9p; and high, with losses at 3p and/or 9p plus losses at 4q, 8p, 11q, 13q, or 17p (Rosin *et al.*, 2000).

I.3.1. Genetic changes on 11q in a variety of cancers

Genetic alterations on chromosome 11q are very common in a variety of cancers. The majority of these genetic changes have been found to be located in two regions: one is at 11q22-25 and the other at 11q13-14 (Table 2).

11q22-25 region. High frequencies of AI have been observed at this region using microsatellite analysis (Table 2). The involvement of several putative tumor suppressor genes has been suggested for this region based on these data. For example, Uzawa indicated in his paper that two putative TSGs might be located at 11q23 and 11q25 and play a role in oral cancer (Uzawa *et al.*, 1996). Herbst reported a high frequency of LOH at 11q23.1-23.2 and 11q23.3 in cutaneous malignant melanoma and suggested putative TSGs in these two loci (Herbst *et al.*, 1999). Koreth proposed two discrete tumor suppressor loci, 11q23.1 and 11q25 (Koreth *et al.*, 1999) based on their data. Finally, Skomedal indicated that a putative TSG at 11q23.1 might be involved in carcinogenesis of cervical cancer (Skomedal *et al.*, 1999). It should be noted that LOH in this region has been shown to correlate with clinical outcome. For example, LOH of 11q22-qter in esophageal squamous cell carcinoma is associated with lymph node metastasis (Tada *et al.*, 2000); LOH at 11q24.1-25 in young woman is associated with poor survival in breast cancer (Gentile *et al.*, 1999); and LOH for 11q23-qter is associated with poor survival in ovarian cancer (Gabra *et al.*, 1995, 1996), etc.

11q13-14 region. There are several critical tumor genes that have been localized in this region and which may play a role in carcinogenesis in a variety of human tumors. By applying microsatellite analysis, a high frequency of LOH was observed for this region in numerous tissues (Table 2). The most important, numerically, are carcinomas of breast and head and neck regions.

Table 2.   **AI on 11q in various cancers as detected by microsatellite analysis**

Tumor types	Location	Cases	Reference
Breast cancer	11q23	326/776 (42%) [a]	Launonen et al., 1999
	11q22-23.1	31/49 (63%)	Koreth et al., 1997
	11q25	23/45 (51%)	Koreth et al., 1997
	11q13	24/36 (67%)	Zhuang et al., 1995
	11q	19/44 (43%)	Hampton et al., 1994
	1122-23	22/57 (39%)	Carter et al., 1994
Cervical carcinoma	11q23	34/81(42%)	Mugica-Van et al., 1999
	11q23.1-23.2	20/33 (60.6%)	Pulido et al., 2000
Ovarian cancer (Early)	11q22-q23	4/13 (31%)	Koike et al., 1999
	11q13	4/16 (25%)	Weitzel et al., 1994
	11q13	72/81(89%)	Dhar et al., 1999
Lung cancer	11q23-q24	20/28 (71%)	Wang et al., 1999
	11q13 (*MEN1*)	4/11 (36%)	Debelenko et al., 1997
Cutaneous malignant melanoma	11q23.1-q23.2	11/44(25%)	Herbst et al., 1999
	11q23.3	7/27(26%)	Pruneri et al., 2000
	11q13	12/48(25%)	Pruneri et al., 2000
B cell pro-lymphocytic leukemia	11q23.1	7/18 (39%)	Lens et al., 2000

[a] Values given as number of samples showing loss/total number of informative cases. Values in parentheses are percentages.

I.3.2. Genetic alterations at 11q in head and neck squamous cell carcinoma (HNSCC)

Genetic changes at 11q are frequently observed in head and neck SCC, including its subset, oral SCC and other aerodigestive tract cancers. Most of the genetic changes are found in two regions: One is at 11q22-23 and the other is at 11q13 (Table 3).

11q22-23 region. To date only a few papers have reported data for this region in HNSCC. Most of these studies have employed the microsatellite assay. Deletion of one or several TSGs is suggested by results obtained with this assay (Nunn *et al.*, 1999; Uzawa *et al.*, 1996; Steenbergen *et al.*, 1995). From the standpoint of association with outcome, AI at 11q22-23 has been found to be associated with lymph node metastasis (Tada *et al.*, 2000) and recurrence (Lazar *et al.*, 1998) in HNSCC.

11q13 region. A high frequency of AI has been reported at multiple loci on 11q13 in HNSCC (Table 3). This suggests that several candidate TSGs are located in this region. Venugopalan suggested the presence of a TSG at *int2-D11S533* in HNSCC (Venugopalan *et al.*, 1998) and Jin, suggested a TSG at 11q13-23 in HNSCC (Jin *et al.*, 1998).

Table 3.   AI at loci on 11q in HNSCCs

Location	Cases	Reference
11q13	13/33 (39%)[a]	Dwight *et al.*, 2000
11q13	6/20 (30%)	Ah-See *et al.*, 1994
11q13(*int2-D11S533*)	9/23 (39%)	Venugopalan *et al.*, 1998
11q13-14	12/15 (80%)	D'Adda *et al.*, 1999
11q23-25	14/25 (56%)	Uzawa *et al.*, 1996
11q23	13/52 (25%)	Lazar *et al.*, 1998
11q14 (*D11S901*)	7/26 (26.9)	
11q22-23 (*D11S2000*)	13/36 (36.1%)	Hui *et al.*, 1996
11q23.2-24 (*D11S934*)	10/29 (34.5%)	
11q23 (*D11S490*) & 11q13 (*int2*)	14/23 (61%)	Nawroz *et al.*, 1994

[a] Values given as number of samples showing loss/total number of informative cases. Values in parentheses are percentages.

I.3.3. Mechanism of alteration at 11q13 and 11q22-23

Mechanism of alteration at 11q13. The alterations at 11q13 are very complicated. Both deletions and amplifications have been observed. Most studies at 11q13 region suggest that amplification rather than deletion is responsible for alterations in this region (Table 4). Some of these studies used fluorescence in situ hybridization (FISH), one of the most efficient and powerful techniques for studying amplification. The advantage of FISH is that it can readily distinguish amplification and deletion by the observation of copy numbers of fluorescence labeled probe in cells in tissue after hybridization with samples. The reported amplification rate is 20% or higher in HNSCC in most studies (Williams et al., 1993; Lese et al., 1995; Meredith et al., 1995; Jin et al., 1998; Muller et al., 1997;Izzo et al., 1998; Wang et al., 1999). These studies also suggested that there is an amplicon, int2-cyclinD1 amplicon that involved several oncogenes (including CCND1/PRAD1, int2/FGF3, HST1/FGF4, EMS1), located at 11q13, which is involved in the amplification.

The amplification and consequent overexpression of critical genes could confer a selective advantage on a cancer cell with such genetic changes. Although amplification is observed frequently in cancer, the precise mechanisms underlying this change are still not well understood (Vogelstein and Kineler, 1998). According to previous studies, the gene amplification can be observed as either extra-chromosomal amplification, in the form of double-minute chromosomes (dmin), or intra-chromosomal amplification, in the

form of homogeneously staining regions (hsrs). In the case of 11q13 amplification in HNSCC, intra-chromosomal amplification at the entopic 11q13 site seems to be the preferred route, as evidenced by the presence of hsrs containing 11q13 sequences localized to chromosome 11 (Shuster *et al.*, 2000; Roelofs *et al.*, 1993; Lese *et al.*, 1995; Jin *et al.*, 1998) or frequent duplication of 11q13 (Jin *et al.*, 1993; Van Dyke *et al.*, 1994; Shuster *et al.*, 2000). A study by Coquelle and his colleagues in 1997 suggested a model, breakage-fusion-bridge (BFB), for intra-chromosomal genes amplification involving an initial distal breakage event, perhaps at a fragile site, followed by sister-chromatid fusion at the break and proximal breakage of the dicentric bridge at mitosis (Coquelle *et al.*, 1997). In 2000, Shuster and his colleagues explained the mechanism of 11q13 amplification in oral SCC by applying the BFB cycle model (Figure 5). According to his study, there is an initial break distal to *CCND1*, possibly at a fragile site that initiates the process, followed by breakage at a fragile site between *RIN1* and *CCND1* which promotes the BFB cycles (Shuster *et al.*, 2000). BFB could cause the amplification of several critical genes at 11q13, including *int2, CCND1, EMS1, GARP*, etc, which play key role in oral cancer carcinogenesis.

Figure 5. The procedures of BFB cycle model of 11q13 amplification (Adapted from Shuster *et al.*, 2000).

A: An initial break distal to *CCND1* could lead to chromosome fusion after replication, resulting in a dicentric chromosome with two copies each of *RIN1* and CCND1.

B: This dicentric chromosome could then undergo one cycle of BFB, with the break occuring between *RIN1* and *CCND1*.

C: Continued cycles of BFB would Lead to amplification of *CCND1*, Flanked single copies of *RIN1*.

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According to Shuster's study, these two fragile sites play critical roles in initiating the BFB cycle to cause the amplification of 11q13, as well as in determining the size and genetic content of amplified units. One possible fragile site involved in gene amplification of 11q13 in oral cancer, *FRA11A*, is found to be located between *RIN1* and *CCND1* (Shuster *et al.*, 2000). Further evidence to support the occurrence of a second break distal to *CCND1* is the observation that 11q13 amplification is usually accompanied by distal deletions between YACs 55G7 and 749G2 (Jin *et al.*, 1998).

Since the genes in an amplicon always amplify together, some believe that samples with *int2* amplification should reflect the amplification of other closely localized genes. Consequently the amplification of *int2* gene has been used as a marker by some to study the amplification of other oncogenes at 11q13, including *cyclin D1* (Izzo *et al.*, 1998; Wang *et al.*, 1999).

However, other individuals have suggested that some of the alterations at 11q13 could involve deletions. These studies used microsatellite analysis and found AI in the 11q13 region, including *int2*, *MEN1*, 11q13.1*PYGM*, *D11S4946* and *D11S913* loci (Bockmuhl *et al.*, 1996; Hui *et al.*, 1996; D'Adda *et al.*, 1999; Bikhazi *et al.*, 2000; Iwasaki, 1996;Chakrabarti *et al.*, 1998; Dwight *et al.*, 2000; Guo *et al.*, 2001; Nord *et al.*, 1999). However, as discussed before, the limitation of microsatellite analysis is that it cannot distinguish deletion from amplification. Ah-See and his co-workers (1994) have shown that PCR-based assays cannot readily distinguish duplication or low-level amplification

of an allele from loss of heterozygosity, particularly if there are contaminations of normal cells within the tumor. Nawroz *et al.* (1994) have stated that the LOH on 11q might in fact represent amplification at this region (11q13). In contrast, in 1994, Ah-See *et al* used microsatellite analysis to score for AI and Southern blots to evaluate the amplification of *CCND1* at the same time. The results demonstrated amplification in only one of 20 tumors but AI in 9 of the 20 tumors. Such results would suggest that loss of TSGs at this region by deletion or mitotic recombination is a more likely scenario than amplification. In 1996, by using microsatellite assay and immunohistochemical analysis, Murali and coworkers found AI at 11q13 in 9 of 23 cancers, but amplification in only one of the 9 cancers, again pointing to a deletion theory.

All these literature suggest that both amplification and deletion occur at 11q13. One possible interpretation is that because TSGs (*MEN1* etc) and oncogenes (*CCND1, INT2, EMS1* etc) are both locate in this region, there might be different pathways of carcinogenesis, either involving amplification of oncogenes at 11q13, or involving deletion of TSGs at 11q13.

No matter whether the changes at 11q13 are deletion or amplification, the alterations have been correlated with clinical outcome, such as poor prognosis (Gebhart *et al.*, 1998; Akervall *et al.*, 1997; Akervall *et al.*, 1995) and metastasis (Alavi *et al.*, 1999). However, although many studies have investigated 11q in cancer, few have examined AI profiles in premalignant lesions, which is an open area for further research, and will be a focus for this thesis.

Table 4.   Amplification at 11q13 as identified with FISH in HNSCC

Location	Cases	Reference
11q13 (*CCND1*)	13/23 (56.5%)[a]	Fujii *et al.*, 2001
11q13	8/20 (40%)	Ott *et al.*, 2002
11q13 (*int2*)	12/21 (57%)	Bayerllein *et al.*, 2000
11q13 (*CCND1*)	5/20 25%)	Alavi *et al.*, 1999
11q13	31/85 (36%)	Williams *et al.*, 1993
11q13	146/282 (52%)	Muller *et al.*, 1997
11q13	22/56 (39%)	Meredith *et al.*, 1995
11q13	11/50 (20%)	Fortin *et al.*, 1997

[a] Values given as number of samples showing loss/total number of informative cases. Values in parentheses are percentages.

Mechanism of alteration at 11q22-23. In contrast, most of studies for the region of 11q22-23 indicate that alterations at 11q22-23 involve deletion. Besides studies using microsatellite analysis (Table 2 and Table 3), there are a few in which FISH technique was used to study this region. Most of these FISH studies were investigating leukemias. Only deletion was observed at the region spanning 11q22.3-11q23 in some of these studies (Leblanc *et al.*, 1996; Dohner *et al.*, 1997; Zhu *et al.*, 1999; Lens *et al.*, 2000; Zhu

et al., 2000; Cuneo *et al.*, 2002). Interestingly, there are five articles that also reported amplification of Myeloid Lymphoid Leukemia gene (*MLL* gene) or 11q23, where the *MLL* gene is located, in leukemias by applying FISH (Michaux *et al.*, 2000; Cuthbert *et al.*, 2000; Streubel *et al.*, 2000; Reddy *et al.*, 2001; Avet-Loiseau *et al.*, 1999).

In HNSCC, there is only one study applied FISH to detect the alteration of 11q22-25 region. In this study, 5/16 (31%) of adenomas and 2/25 (8%) of primary hyperplasia were observed deleted at 11q23, but no amplification was observed. To date, no one has reported amplifications or oncogenes located at 11q22-25 involved in HNSCC.

1.3.4. 11q alterations in head and neck premalignant lesions

Most of previous studies of 11q reported that alteration at 11q occurred late in carcinogenesis, between severe dysplasia and SCC (Table 2). So far, few papers have investigated 11q alterations in head and neck premalignant lesions. By applying microsatellite analysis, el-Naggar *et al.* (1995), Califano *et al.* (1996) and Poh *et al.* (2001) have demonstrated AI at 11q in head & neck premalignant lesions (Table 5). In 2000, Rosin *et al.* (2000) indicated that LOH at 11q was strongly associated with an increase risk for progression. Their *P*-value was 0.062 and 0.011 for hyperplasia and low-grade dysplasia separately. As we know, there are two hot spots at 11q, 11q13 and 11q22-23 regions, which play different roles in oral carcinogenesis. The limitation of this study is that it scored AI at these two sites together. This study needs to be confirmed using a larger number cases and scoring the two sites at 11q separately.

Amplification at 11q13 has also been observed in head & neck premalignant lesions. In 1998, Izzo used FISH to show that 7/9 (77.7%) of dysplasia had amplification of *CCND1* (Izzo *et al.,* 1998). In one study, amplification of *int2* gene (also done with FISH) was observed in 1/4 (25%) during hyperplasia to dysplasia transition and 2/4 (50%) of dysplasia (Roh *et al.,* 2000). These two papers suggested that the *int2-cyclinD1* amplicon was amplified in the early stage in carcinogenesis of HNSCC and that *cyclinD1* might be involved in early regulation. However, the case numbers in both of these two papers are

very small and further study is required to confirm the results.

These few papers demonstrate one or more of the following limitations: (1) they had limited number of cases; (2) they grouped all premalignant lesions together without separating them into different stages (e.g. different degree of dysplasia) of premalignant lesions, and (3) they scored genetic alterations at 11q13 and 11q23 together (Table 5). It is still not well understood whether gene alterations at 11q precede carcinoma development or results from the unstable nature of tumors. Based on these limitations, more studies of genetic alterations at 11q in OPL need to be done to better understand the timing and mechanism of alterations of 11q in OPL and their clinical value.

Table 5. **Genetic alterations at 11q of head and neck premalignant lesions**

Analysis applied	Location	Stages	Frequency	Reference
Microsatellite analysis	11q	Premalignant lesions	9/31 (29)[a]	Califano et al., 1996
Microsatellite analysis	11q	Premalignant lesions	15/75 (20)	Rosin et al., 2000
Microsatellite analysis	11q	Non-invasive lesion	1/15 (7)	el-Naggar et al., 1995
Microsatellite analysis	11q13-22	Hyperplasia	2/44 (5)	Poh et al., 2001
		Low-grade dysplasia	6/52 (12)	
		High-grade dysplasia	6/37 (16)	

[a] Values given as number of samples showing loss/total number of informative cases. Values in parentheses are percentages.

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I.3.5. Main genes on chromosome 11q

Table 6 contains a list of genes that are referred to in articles on 11q13 as possible oncogenes/TSGs in that region. At 11q23.1, the genes myeloid lymphoid leukemia gene (*MLL* gene), *ALL-1*, and Homology of Trithoraz (*HRX*), are thought to play an important role in hematogenous malignancies with *ATM* being a key candidate for mutation in solid tumors, including HNSCC. A brief discussion of seven of these genes is given below.

Table 6.   List of genes located at 11q13

	GENE	Location	Function
CCND1	*Cyclin D1*	54917530-54930900	Regulating progress through the cell cycle
FGF4/HSTF1	Fibroblast growth factor 4 precursor	55049454-55051829	Heparin-binding growth factor
FGF3/int2		55086382-55087115	Stimulate the proliferation of fibroblast and endothelial cells
FADD	Fas associated via death domain	55347924-55351947	Universal adapter protein in apoptosis that mediates signaling of all known death domain-containing members of the TNF receptor superfamily
EMS1	Cortactin	55543127-55581159	Involved in the restructuring of the cortical actin cytoskeleton
RIN1	*ras* inhibitor	57291330-57295778	RAS inhibitor
MEN1	Multiple endocrine neoplasia 1	59347865-59355064	Mutated form is responsible for Multiple endocrine neoplasia
VEGFB/VRF	Vascular endothelial growth factor	59706252-59709238	Vascular endothelial growth factor

	GENE	Location	Function
PPP1CA/PPP1 A		61059778- 61063386	Serine/threonine-specific protein phosphatases, A candidate TSG
GST-pi	Anionic glutathione S-transferase	61182590- 61185105	Xenobiotic detoxification
CPT1A	Liver carnitine palmitoyltransferase I	62546480- 6260940	Key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane
NUMA1		75601775- 75679417	Cell-cycle related protein
PAK1	p21-activated kinase 1	81163598- 81233157	Regulate cytoskeletal dynamics by decreasing MLCK activity and myosin light-chain phosphorylation
GARP	Glycoprotein A repetitions predominate	81886288- 81898610	A candidate oncogene at 11q13.5-11q14

Location: From Human Genome project draft (UCSC) Dec. 22, 2001 browser (http://genome.cse.ucsc.edu/index.html).

I.3.5.a.   CCND1 (cyclin D1, PRAD1) gene at 11q13

The fidelity of cell division requires that an accurate copy of a complete genome be passed on to each daughter cell. This means that earlier events in the cell cycle, such as completion of DNA replication, must be accomplished for later events such as mitosis and cytokinesis to occur. Eukaryocytic cells have developed feedback controls called checkpoints to monitor and regulate various steps in cell-cycle progression.

The CCND1 gene (cyclinD1 gene) is a proto-oncogene that codes for a protein that is strongly implicated in cell cycle control. CCND1 binds to and activates a cell cycle kinase that controls phosphorylation of the retinoblastoma protein (pRb) (Donnellan et al., 1998). Phosphorylation to Rb gene causes it to release proteins such as transcription factor E2F. This factor can in turn bind to regulatory region in the DNA for specific genes and promote their transcription. This in turn drives cells through the checkpoint in late G1 phase.

Substantial evidence suggests that the level of cyclin D1 protein is critical to proper cell cycle progression and that deregulated expression of this gene may disrupt cell cycle control and contribute to genomic instability (Almasan et al., 1995). Indeed, cells overexpressing cyclin D1 have a shortened G1 phase, reduced dependency on exogenous mitogens, and abnormal proliferative characteristics (Quelle et al., 1993; Jiang et al.,

1993). These cells also demonstrate a higher frequency of gene amplification, especially

under conditions of genotoxic stress (Zhou *et al.*, 1996). Moreover, overexpression of

cyclin D1 has been shown to be associated with tumor transformation in *in vitro* studies

in normal fibroblasts and primary embryo cells transfected with this gene (Jiang *et al.*,

1993; Hinds *et al.*, 1994; Uchimaru *et al.*, 1996), and increased tumorigenesis in *in vivo*

studies using transgenic mice that over-express this protein (Wang *et al.*, 1994).

CCND1 is commonly found amplified and overexpressed in almost all types of cancers,

such as lung cancer (Reissmann *et al.*, 1999), ovarian cancer (Dhar *et al.*, 1999),

myeloma (Hoechtlen-Vollmar *et al.*, 2000), colorectal cancer (Mckay *et al.*, 2000),

prostate cancer (Kaltz-Wittmer *et al.*, 2000), mantel-cell lymphoma (Remstein *et al.*,

2000), breast cancer (Rennstam *et al.*, 2001), and HNSCC (Julie *et al.*, 1998).

I.3.5.b.   int2 (FGF3) gene at 11q13

The gene *int2* is also located in the chromosome 11q13 region. It is the first oncogene

recognized as a member of fibroblast growth factors (FGFs). Activation of the *int2* gene

is a result of transcriptional deregulation, resulting in constitutive overexpression of the

normal polypeptide products. The gene *int2* encodes a growth factor known as FGF3,

which is structurally related to other FGF family proteins and participates in several

biological processes such as cell differentiation, motility, proliferation and angiogenesis

(Dickson and Peters, 1987). All FGFs, including FGF3, have oncogenic potential. They

are synthesized by many tumor cells and can induce blood vessel formation (Burgess and Maciag, 1989; Goldfarb, 1990). Transferring *int2* gene into mice results in hyperplasia of the mammary gland and prostate; however, tumor formation is rare (Muller *et al.*, 1990; Ornitz *et al.*, 1991).

Genetic alteration of *int2* has been reported mainly in HNSCC and breast cancer (Watatani *et al.*, 2000; Selim *et al.*, 2001, 2002; Lese *et al.*, 1995; Rubin *et al.*, 1995). A few papers also reported alteration of *int2* in prostate carcinomas (Latil *et. al.*, 1994) and ovarian cancer (Foulkes *et al.*, 1993). In HNSCC, a few papers reported alteration at *int2* as deletion (Friedman *et al.*, 1989; Thakkar *et al.*, 1989; Ah-See *et al.*, 1994; Hui *et al.*, 1996). In contrast, alterations at *int2* were recognized as amplification by more papers (Williams *et al.*, 1993; Lese *et al.*, 1995; Rubin *et al.*, 1995; Muller *et al.*, 1997; Jin *et al.*, 1998; Wang *et al.*, 1999; Shuster *et al.*, 2000; Roh *et al.*, 2000).

I.3.5.c.   *HST1 gene at 11q13*

HST1 (Heparin secretory transforming factor 1, *FGF4*) is a human transforming gene originally detected by NIH 3T3 fibroblast transfection with DNA from human stomach tumors (Terada *et al.*, 1986). It also belongs to the fibroblast growth factor family. Activation of *HST1* is a result of transcriptional deregulation, resulting in constitutive overexpression of the normal polypeptide products.

In HNSCC, *HST1* has been reported to be co-amplified with some genes at 11q13 (Sugimura *et al.,* 1990; Lese *et al.,* 1995; Meredith *et al.,* 1995; Shuster *et al.,* 2000). It is believed to be involved in HNSCC carcinogenesis (Lese *et al.,* 1995) and angiogenesis (Schweigerer, 1989).

I.3.5.d. *EMS1 gene at 11q13*

EMS1 [mammary tumor and squamous cell carcinoma-associated (p80/85 src substrate)] gene has been mapped to 11q13 (Schuuring *et al.,* 1992). It encodes human cortactin, an acting-binding protein possibly involved in the organization of the cytoskeleton and cell adhesion structures (Shuster *et al.,* 2000). Since amplification of the 11q13 region has been associated with an enhanced invasive potential of these tumors (Adnane *et al.,* 1989; Borg *et al.,* 1991; Kitagawa *et al.,* 1991; Schuuring *et al.,* 1993), overexpression and concomitant accumulation of the *EMS1* protein in the cell-substratum contact sites might contribute to the invasive potential of these tumor cells.

EMS1 is generally believed to be an oncogene (Schuuring, 1995; Jin *et al.,* 1998). Amplification of *EMS1* gene has been observed in breast cancer (Schuuring *et al.,* 1992; Brookes *et al.,* 1993) and HNSCC (Williams *et al.,* 1993; Jin *et al.,* 1998; Shuster *et al.,* 2000). Moreover, in HNSCC, 11q13 amplification, including *EMS1,* is associated with poor prognosis (Meredith *et al.,* 1995; Rodrigo *et al.,* 2000).

1.3.5.e.   MEN1 gene at 11q13

Multiple endocrine neoplasia type1 (*MEN1*) is an autosomal dominant disorder that is associated with endocrine tumors of the parathyroid, the endocrine tissues, and the anterior pituitary (Wermer, 1954; Weber *et al.*, 1994). Additional associations include foregut carcinoid, facial angiofibroma, and lipomas.

Larsson *et al.* (1998) initially made the critical observation that two malignant insulinomas from brothers with *MEN1* showed loss of the entire copy of chromosome 11q that was inherited from their parents without *MEN1*. This study suggested that the wild type *MEN1* gene functions as a TSG. *MEN1* was mapped to chromosome 11q13 in later studies (Nakamura *et al.*, 1989; Bystrom *et al.*, 1990; Janson *et al.*, 1991). Other evidence supporting a role for *MEN1* as a tumor suppressor gene comes from microsatellite studies that show LOH of the normal allele at the *MEN1* locus (Vogelstein and Kinzler, 1998). Depending on the probes used, LOH has been shown to be frequent in *MEN1* tumors of the parathyroid, approaching 100% (Lubensky *et al.*, 1996; Zhuang *et al.*, 1995). 11q13 LOH has also been found in 85% of nongastrinoma pancreatic islet tumors and in 40% of gastrinomas (Debelenko *et al.*, 1997).

I.3.5.f.   RIN1 gene at 11q13

Studies using FISH on 10 oral SCC cell lines, which have been identified with

amplification of *int2*, *HST1* and *CCND1* shown that *RIN1* (Ras interaction/interference) gene is co-amplified (7 out of 10 cell lines) with other critical oncogenes, such as *int2*, *CCND1* and *HST1* in oral cancer (Shuster *et al.*, 2000). Human *RIN1* was first characterized as a RAS (an oncoprotein) binding protein based on the properties of its carboxyl-terminal domain (Han *et al.*, 1997). Through a separate domain, *RIN1* has also been shown to interact with and serve as a substrate for the tyrosine kinase ABL (an oncoprotein) (Afar *et al.*, 1997)). The intimate relationship of *RIN1* with both RAS and ABL oncoproteins suggests the possibility of a direct or indirect role for *RIN1* in naturally occurring tumors (Shuster *et al.*, 2000).

I.3.5.g.   *ATM gene at 11q23.1*

ATM (Ataxia Telangiectasia Mutated) gene was found to be located on chromosome band 11q23.1 and its mutated form is responsible for ataxia telangiectasia (AT).

The ATM protein plays a key role in signaling cell cycle arrest in response to DNA double-strand breaks (Kastan *et al.*, 1992; Meyn *et al.*, 1994). Without this surveillance mechanism, cells are prone to replicate damaged DNA templates in S-phase and segregate damaged chromosomes through mitosis (Meyn *et al.*, 1995). ATM protein also has been shown to interact with other proteins (Shafman *et al.*, 1997) and to play a role in controlling cell cycle and apoptotic pathway (Barlow *et al.*, 1997) as well as signal transduction (Keegan *et al.*, 1996). Wild-type ATM protein is required for up-regulation

of p53 tumor suppressor protein in response to ionizing radiation and other DNA damaging agents (Westphal *et al.*, 1997; Hawley *et al.*, 1996). Cells lacking the ATM protein show a reduced and delayed activation of the tumor suppressor gene *p53* in response to DNA damage. The proposed function of the ATM protein points to a potential role of *ATM* as a tumor suppressor gene.

AT is a hereditary autosomal recessive disorder with a variety of different clinical manifestations, including progressive cerebellar ataxia, oculocutaneous telangiectasis, immunodeficiency, chromosome instability, radiation sensitivity and an increased susceptibility for the development of various malignancies (Gatti *et al.*, 1991). The incidence of the disease is estimated to be 1/40,000-100,000 (Telatar *et al.*, 1998; Gatti *et al.*, 1991; Sedgwick *et al.*, 1991). AT patients also exhibit severe hypoplasia of the thymus and lymphoid tissues, moderate to severe hypogonadism and atrophy of the cerebellum. An estimated 1 in every 100 AT children from the age of 10 onward will develop a new cancer each year (Morrell *et al.*, 1986; Taylor, 1992). The risk of developing cancer is 61 to 184 times higher in AT homozygotes than in the general population.

More than 250 mutations in the *ATM* gene have been identified in AT families (Vogelstein and Kinzler, 1998). LOH of *ATM* has been reported in breast cancer (Waha *et al.*, 1998), cervical cancer (Skomedal *et al.*, 1999), adult acute lymphoblastic leukemia (Haidar *et al.*, 2000), ovarian cancer (Launonen *et al.*, 1998), lung cancer (Murakami *et al.*, 1999), and also HNSCC (Lazar *et al.*, 1998).

Although these data suggest the possibility that the *ATM* gene could be acting as a TSG for these cancers, definitive proof is not available. For HNSCC, there have been no reports of mutation in this gene.

II. STATEMENT OF PROBLEMS

II.1. Where are the additional tumor genes at 11q13 located?

Although a number of tumor genes including *CCND1* and *int2* have been established in the 11q13 region, additional tumor genes, particularly tumor suppressor genes, are suspected to be located at the region. It has been proposed that there are many additional genes in the 11q13 region, and that this region may play a key role in controlling chromosomal instability and progression of tumors (Bekri *et al.*, 1997, Izzo *et al.*, 1998, 1999; Zhou *et al.*, 1996; Gebhart *et al.*, 1998). Identification and location of these potential genes will contribute to the understanding of tumorigenesis. A major problem in using tumors to locate tumor genes is that cancers are most often accompanied by an intrinsic genetic instability that results in a cascade of gene alterations in the tumor, many of which are just random changes that are not critical to tumor development.

This thesis used a spectrum of tissue samples including SCC, dysplasia and hyperplasia to search for novel regions of alterations in the 11q13 region. The rationale for using preinvasive lesions is that alterations occurring in these early lesions are more likely to be a driving force for carcinogenesis rather than due to the random genetic instability seen in later lesions including tumors. Consequently, studies of preinvasive lesions are more likely to reveal the small region of loss that contains critical tumor genes.

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II.2. At what stages of oral cancer development does AI occur for *int2* (11q13), *D11S1778* (11q22.3) and any novel loci identified in this study?

Information obtained from allelic imbalance studies has dual merit. The finding of frequently lost regions during cancer development can lead to discovery of new TSGs. In addition, allelic imbalance findings can provide critical information on the role of the presumptive tumor genes even before the cloning of the tumor gene. For example, allelic imbalance studies have shown that there are three discrete regions of loss at 3p, suggesting that each of these three regions contains at least one tumor suppressor gene (Partridge *et al.*, 1996). While we have yet to identify the genes involved at 3p, studies on the timing of such loss during histological progression have already lead to the conclusion that loss on at least one of the regions, 3p14, is an early critical event for cancer development. As discussed in section I.2.3.f., LOH at 3p14 can serve as an important molecular marker for predicting risk of malignant transformation for oral premalignant lesions (Rosin *et al.*, 2000).

While the mapping studies in this thesis may yield information on location of putative tumor suppressor genes at 11q13-14, it may be some time before such genes can be identified. Acquisition of information on at what stage of oral cancer development the new candidate gene is altered will not only provide information on the possible roles of

the gene, but may also facilitate the process of identification of the gene.

In addition to doing temporal studies for any novel loci showing frequent AI in the 11q13-14 region, this thesis will further explore the timing of loss for the 2 regions previously studied in this lab (I.2.3.f.). These regions are defined by markers *int2* which amplifies sequence with the *int2* gene at 11q13 and by marker *D11S1778*, which amplifies sequence that is 0.6 Mb from the *ATM* gene at 11q22.3. Our early studies fused the data from these two loci (I.2.3.f.). These studies suggested that alteration to 11q13-22.3 occurred with formation of SCC. In this thesis, the 2 regions will be examined independently to confirm the association with histological progression.

III. OBJECTIVES

To use microsatellite analysis to examine DNA extracted from severe dysplasia, carcinoma *in situ* (*CIS*) and SCC for novel alterations in the 11q13 region. This region should be distinct from the *int2* (11q13) and *D11S1778* (11q22-23) regions of alteration previously studied in this laboratory.

If a novel region of loss is identified, to determine at what stage of oral cancer development the alteration occurs by performing microsatellite analysis on a spectrum of stages of oral premalignant lesions (hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, *CIS*) as well as invasive SCC.

To determine at what stage of oral cancer development the *int2* (11q13) and *D11S1778* (11q22.3) alteration occur by performing microsatellite analysis of the same oral premalignant and malignant lesions and compare the data obtained with that seen for any novel regions of loss identified in this study.

To determine the significance of LOH at *int2* (11q13) and *D11S1778* (11q22.3) loci to the progression of oral premalignant lesions by comparing frequencies of loss for different locus in low-grade dysplasia with known outcome, i.e., low-grade lesions that did not progress into cancer with morphologically similar lesions that did develop into SCC.

IV.   HYPOTHESES

Microsatellite analysis of microdissected oral premalignant and malignant samples will reveal novel loci in the 11q13 region, which harbors one or several putative tumor genes.

Allelic loss at *int2* (11q13) and *D11S1778* (11q22-23) occur mainly at the tumor stage.

Progressing low-grade lesions (those without dysplasia or with low-grade dysplasia) have increased frequencies of allelic imbalance at *int2* (11q13) and *D11S1778* (11q22-23) in this thesis compared to morphologically similar non-progressing lesions, suggesting a role for this alteration in cancer progression.

V. MATERIALS AND METHODS

V.1. Sample collection

This thesis used paraffin-embedded archival samples from the provincial Oral Biopsy

Service of British Columbia, located at the Oral Pathology Division of Vancouver

General Hospital and Health Sciences Center. This service receives more than 3,500

biopsies of oral lesions received per year (19 years archived). This provides a large

collection of early lesions that can be followed over time. The use of these samples was

approved by the University Ethics Committee.

V.2. Sample sets

Two different sample sets were used.

The first set had 4 groups in it, as follows: primary SCC, severe dysplasia/*CIS*,

mild/moderate dysplasia and hyperplasia (Table 7).

Table 7.   Histological groups in sample set 1

Lesion type	Number of cases
Hyperplasia (without dysplasia)	33
Mild & moderate dysplasia (low-grade dysplasia)	54
Severe dysplasia & *CIS* (high-grade dysplasia)	56
Primary SCC	91
Total	234

The second sample set included 2 groups of cases. Both groups included cases with hyperplasia (without dysplasia), mild dysplasia and moderate dysplasia (Table 8). In 1 group (called "progressing lesions") the lesions later progressed to *CIS* or SCC. No progression occurred in the second group (called "non-progressing lesions").

Table 8.   Histological groups in sample set 2: the progression test series

Lesion Type	Lesions not progressed to *CIS* or SCC	Lesions later progressed to *CIS* or SCC
Epithelial hyperplasia (without dysplasia)	33	6
Mild dysplasia	31	9
Moderate dysplasia	23	14

The criteria for choosing samples for the non-progressing group included confirmation of histological diagnosis by two pathologists using criteria established by the World Health Organization (WHO collaborating Reference centre 1978) and the provision that the sample was large enough to yield sufficient DNA from both the epithelium and from the connective tissue for multiple microsatellite analyses. The third criterion was confirmation that these patients had no prior history of head and neck cancer and, for hyperplasia and dysplasia, that they did not subsequently develop such cancer. This confirmation was obtained from hospital records and by using a computer linkage with the British Columbia Cancer Registry. All but three of these cases had at least 3 years of follow up.

The inclusion criteria for progressing group included confirmation of histological

diagnosis by 2 pathologists, sufficient sample size, and no prior history of HNSCC. A

final provision was that both the primary hyperplastic or dysplastic lesions and their

matching *CIS* or SCC had to be from the same anatomical site as recorded on pathology

reports and patients charts and the interval between the primary lesions and later *CIS* or

SCC had to be longer than 6 months. The later criterion was used to exclude cases where

the appearance of the *CIS* or SCC might be due to inadequate biopsy or sampling of the

index (first) biopsy error. The time interval chosen for exclusion was arbitrary.

V.3. Diagnostic criteria for the samples

The WHO diagnostic were used, which have been reviewed in section I.2.2.b. The

diagnosis was confirmed independently by Dr. R. Priddy and Dr. L. Zhang, oral

pathologists at the BC provincial biopsy service. Only those cases in which the two

pathologists agreed on the diagnosis were used for the study.

V.4. Clinical information

The following clinical data were obtained for the cases studied by examining pathology

reports and hospital charts: smoking habit, age and gender of the patients and anatomical

location of the lesions. Some of this information was not recorded for some cases (see Result section).

V.5. Slide preparation

Tissue blocks for cases were chosen for study removed from the archive and one 5-micron-thick section was cut from each block, stained with H&E (hematoxylin and eosin) and coverslipped for reference. Further sections for microdissection were then cut at a 10 to 12 microns thickness with approximately 15 sections per sample. These sections were also stained with H&E but left uncoverslipped. The H&E procedure is described below:

1. Slides were baked at 37°C overnight in an oven, then at 60 to 65°C for 1 hour, and left at room temperature to cool.

2. Samples were deparaffinized by two changes of xylene for 15 minutes.

3. Dehydration in gradient alcohols (100%, 95, 70% ethanol).

4. Hydration by rinsing in tap water.

5. Slides were placed in Gill's Hematoxylin for 5 minutes then rinsed in tap water.

6. "Blued" with 1.5% (w/v) sodium bicarbonate, then rinsed in water.

7.   Slides were lightly counterstained with eosin, dehydrated, and cleared for
      coverslipping.

8.   Thick sections to be dissected were stained by the above procedure
      without the dehydration step then air-dried prior to microdissection.

V.6. Microdissection

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

V.7. Sample digestion and DNA extraction

The microdissected tissue was collected in a 1.5 ml eppendorf tube and digested in 300 µl
of 50 mM Tris-HCL (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and
proteinase K (0.5 mg/ml) at 48°C for 72 or more hours. During incubation, samples were
spiked with 10 or 20 µl of fresh proteinase K (20 mg/ml) twice daily. The DNA was then

extracted 2 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 quantification (Rosin *et al* 1997; Zhang *et al* 1997).

V.8. DNA quantification

Fluorescence analysis with a Picogreen kit (Molecular Probes, Eugene, Oregon) was used to quantify DNA. This method used 2 standard curves. The low concentration standard curve was used for samples with 1 to 20 ng/μl, while the high concentration standard curve was used for concentrations between 10 and 400 ng/μl. Absorbance was read with a SLM 4800C spectrofluorometer (SLM Instruments Inc. Urbana, IL). The sample DNA concentration was then determined from one of the standard curves depending on its concentration, hence absorbance. A series of dilutions were done subsequently to adjust the concentration of DNA to 5 ng/μl with LOTE buffer (Rosin *et al* 1997; Zhang *et al* 1997).

V.9. Primer extension preamplification (PEP)

If the concentration of DNA was low (less than 100 ng total), a procedure called PEP was performed. PEP involves amplification of multiple sites of the genome using random

primers and low stringency conditions, thus increasing the amount of total DNA for

microsatellite analysis. The PEP reaction was carried out in a 60 µl reaction volume

containing 20 ng of the DNA sample, 900 mM of Tris-HCL, (pH 8.3), 2 mM of dNTP

where N is A, C, G and T, 400 µM of random 15-mers (Operon Technologies,

California), and 1 µl of Taq DNA polymerase (Introgen, Gibco). 2 drops of mineral oil

were added prior to the reaction. The amplification was done on an automated thermal

cycler (Omigene HBTR3CM, Hybaid Ltd.) and 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).

V.10. Coding samples

All samples were coded in such a way that the analysis of allelic imbalance would be

performed without the knowledge of the sample diagnosis.

V.11. End-Labeling

One more step prior to microsatellite analysis was end-labeling of one member of the

chosen microsatellite primer pair. Those reactions were carried out in a total volume of

50 µl which contained 38 µl of PCR grade water, 5 µl of 10 × buffer for T4

polynucleotide kinase (New England BioLabs, Beverly, MA), 1.2 µl of 100 × BSA, 100

ng of one of the primer pair, 3 μl of T4 polynucleotide kinase (New England BioLabs, Beverly, MA), and 2 μl of [γ-^{32}P] ATP (20 μCi, Amersham). The PCR reaction was 1 cycle at 37°C for 60 min run on the thermal cycler (Rosin *et al* 1997; Zhang *et al* 1997).

V.12. Microsatellite analysis: PCR amplification

The microsatellite markers came from Research Genetics (Huntsvile, AL) and mapped to the following regions: 11q13 (*D11S4207*, *D11S916* and *int2*) and 11q23 (*D11S1778*). Markers *int2* was used to amplify DNA sequence within the *int2* gene (need to confirm). Marker *D11S1778* is 0.6 Mb from the *ATM* gene and has been used as the marker for *ATM* gene in many publications (Laake K *et al.*, 1997; 1999).

PCR amplification was carried out in a 5 µl 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 (Invtogen, Gibco), PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH8.8), 6.7 mM magnesium chloride, 10 mM (β-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide], and 2 drops of mineral oil. Amplification involved 1 cycle of pre-heat at 95C for 2 min; 40 cycles of 1) denaturation at 95 C° for 30s, 2) annealing at 50-60 C° (depending on the primer used) for 60s, and 3) polymerization at 70 C° for 60s; and 1 cycle of final polymerization at 70 C° for 5 min. The PCR products were then diluted 1:2 in loading buffer and separated on 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography. The films were coded and scored for allelic imbalance (Zhang *et al.*, 1997).

V.13. Scoring of allelic imbalance

For informative cases (meaning both alleles are of different length and thus could be distinguished from one another by electrophoresis), allelic loss is scored if the signal intensity of the band is at least 50% less than its normal control counterpart (connective tissue DNA) (Rosin *et al* 1997; Zhang *et al* 1997). All samples showing allelic imbalance were subjected to repeat analysis after a second independent amplification and re-scored whenever the quantity of DNA is sufficient.

VI. RESULTS

VI.1. Choice of microsatellite markers for this study

There is strong evidence supporting the involvement of alterations in the 11q13 region in cancer development and the potential involvement of numerous genes has been suggested (see Table 6 for list). However, with the exception of *CCND1*, the evidence for a role of these genes in oral carcinogenesis is largely speculative. When the research in this thesis was first started, a decision was made to focus on the DNA sequence between *cyclin D1* (*CCND1*) and *int2* in the 11q13 region, since many of these putative oncogene/TSGs mapped to that region, and to use the microsatellite assay to screen for novel regions of alterations (distinct from *CCND1*). The rationale was that such novel alterations could be used to localize genes playing a role in oral carcinogenesis. The Human Genome Database (http://www.gdb.org/) was used to identify potential microsatellite markers between *CCND1* and *int2*. Of the markers chosen for the study, *D11S4207* (*AFMa103zf9*) proved to be highly informative with frequent allelic loss in tumor DNA from microdissected oral SCC samples. It was thus chosen as a starting point for the study and the majority of the work described below used this marker. It should be noted that during the course of this research, there has been a vast improvement in sequence analysis for the genome. As shown in Figure 6, this has resulted in the current localization of *D11S4207* to a region that is 20 Mb telomeric to the *CCND1-Int2* region

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[sequence localization by University of California Santa Cruz database (http://genome.cse.ucsc.edu/index.html) using the Human Genome project draft (UCSC) dated Dec. 22, 2001]. As shown below, this marker identifies a novel region of frequent alteration in oral SCC and premalignant lesions that occurs independent of alterations at *CCND1-Int2*. The evidence in support of this statement is given below.

Figure 6. Microsatellite results for cases with AI at *D11S4207* and retention at *int2*.

A: Primary SCC;
B: High-grade dysplasia;
C: Low-grade dysplasia;
D: Hyperplasia

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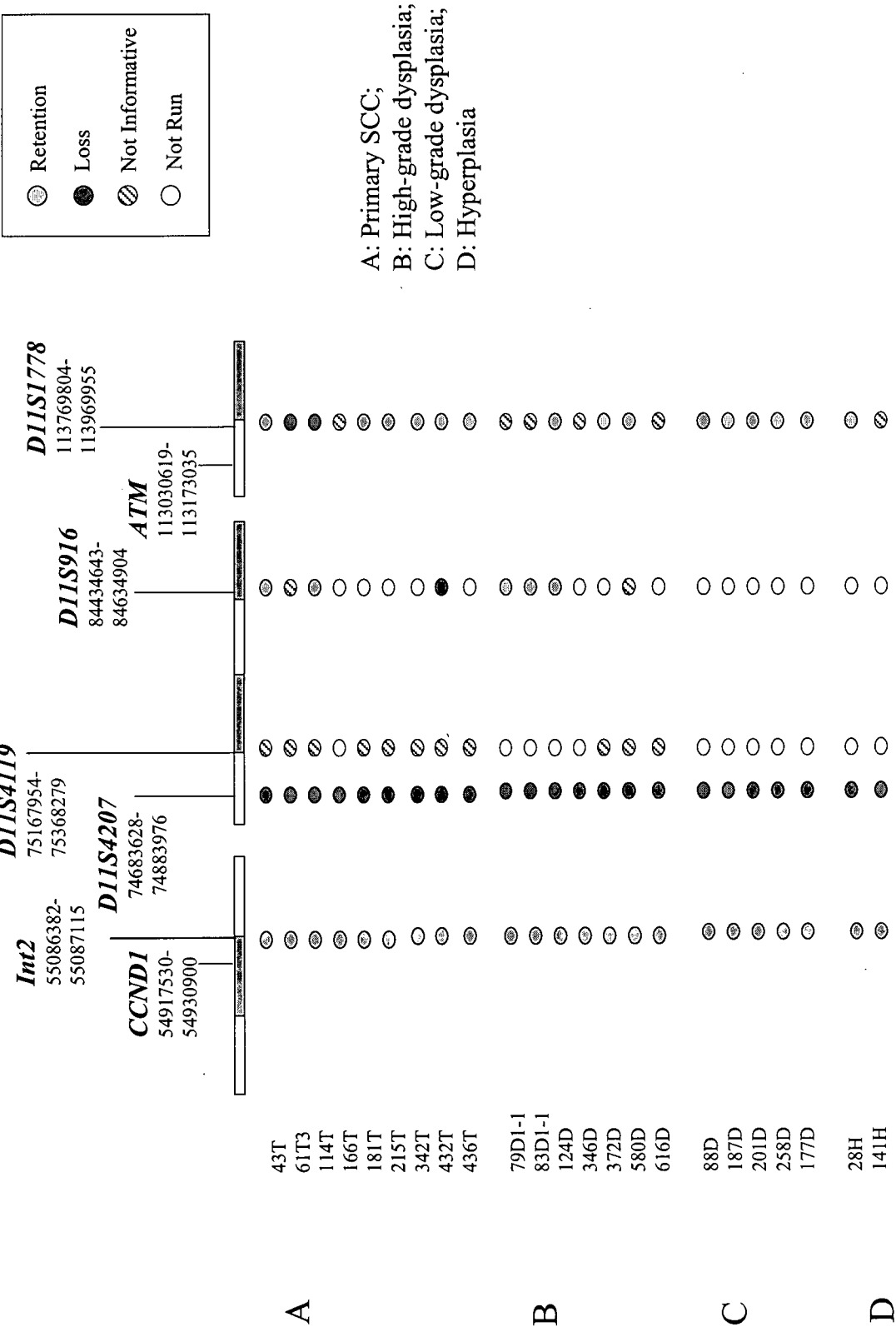
VI.2. AI at *D11S4207* in oral SCCs and premalignant lesions

In order to determine the frequency of AI at *D11S4207*, tumor cells were isolated by microdissection from archived paraffin blocks of 91 cases of oral SCC. The DNA was extracted and the locus was amplified using the *D11S4207* primers. The underlying stroma served as a source of normal control DNA. The amplified products were separated on polyacrylamide gels. 74 (81%) of these cases were informative (showed 2 bands), 35 (47%) of which showed AI. A picture of the alteration in band intensities (AI) at this locus is shown for several cases in Figure 7.

Figure 7.   Microsatellite analysis of SCC cases at *D11S4207* and *int2*
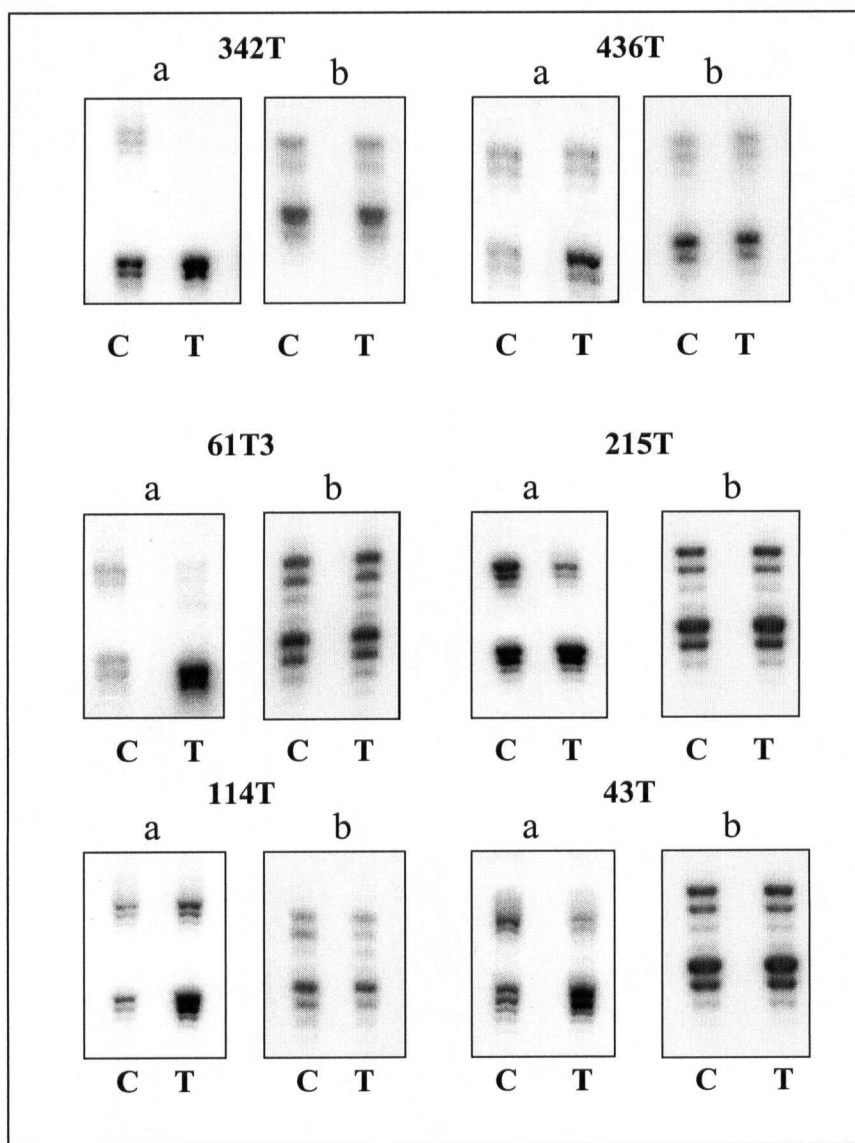
D11S4207 and *int2* were amplified from areas of epithelium of SCC (T) and normal connective tissue (C). a: Images showing AI at *D11S4207; b*. Images showing retention at *int2*.

To determine whether this alteration was also frequent in premalignant lesions, the same primers were used to amplify DNA isolated from 33 epithelial hyperplasias, 54 low-grade dysplasias and 56 high-grade dysplasias. It should be noted that these samples all came from cases in which there was no prior history of oral cancer (i.e. primary lesions). The data is shown in Table 9.

Table 9.   **Allelic imbalance at *D11S4207* in a spectrum of primary lesions with different histological diagnoses**

Diagnoses	Number of cases	Informativity[a]	AI[b]
Hyperplasia	33	19/33 (58)	7/19 (37)
Low-grade dysplasia	54	29/54 (54)	10/29 (34.5)
High-grade dysplasia	56	38/56 (68)	18/38 (47)
SCC	91	74/91 (81)	35/74 (47)

[a]Informativity: Number of cases informative for this locus (showing 2 bands)/total case number. Numbers in parentheses are percentages.

[b] Number of cases showing AI/total number of informative cases. Numbers in parentheses are percentages.

High frequency of AI at *D11S4207* was present in hyperplasia cases (37%) and low-grade dysplasia cases (34.5%) and slightly elevated in high-grade dysplasia (47%) and SCC cases (47%). Figure 8 provides examples of band appearance of gels showing DNA from hyperplasias with AI. Figure 9 compares the frequencies observed at this locus with those seen with microsatellite markers at 3p14 (*D3S1228, D3S1234, D3S1285* and *D3S1300*) and 9p21 (*INFA, D9S1751, D9S171* and *D9S1748*). The latter regions are those that have been previously reported to be among the earliest alteration in oral carcinogenesis (see section I.2.3.f. for evidence). As a comparison, AI at 8p (*D8S261, D8S262, D8S264*) is infrequent in hyperplasias, and both low-grade and high-grade dysplasia but increases significantly in SCCs (see section I.2.3.f. for evidence).

Figure 8. **Microsatellite analysis of hyperplasia cases at *D11S4207***
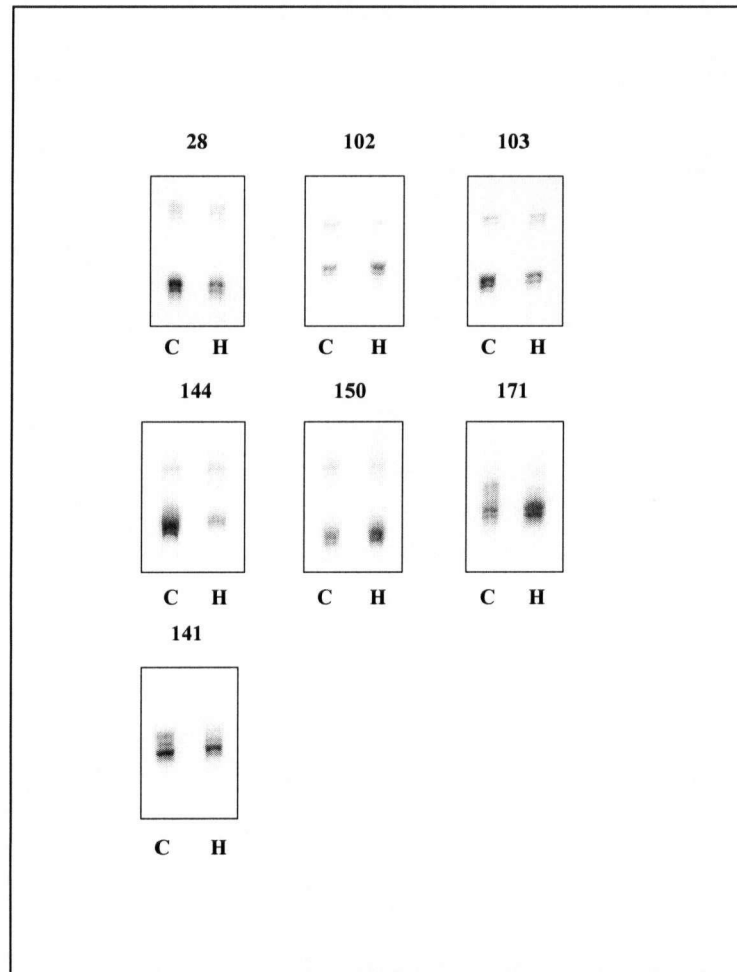
D11S4207 were amplified from areas of epithelium of hyperplasia (H) and normal connective tissue (C).

Figure 9.   **Comparison of AI frequencies observed at *D11S4207* with those at**

3p14, 9p21 and 8p
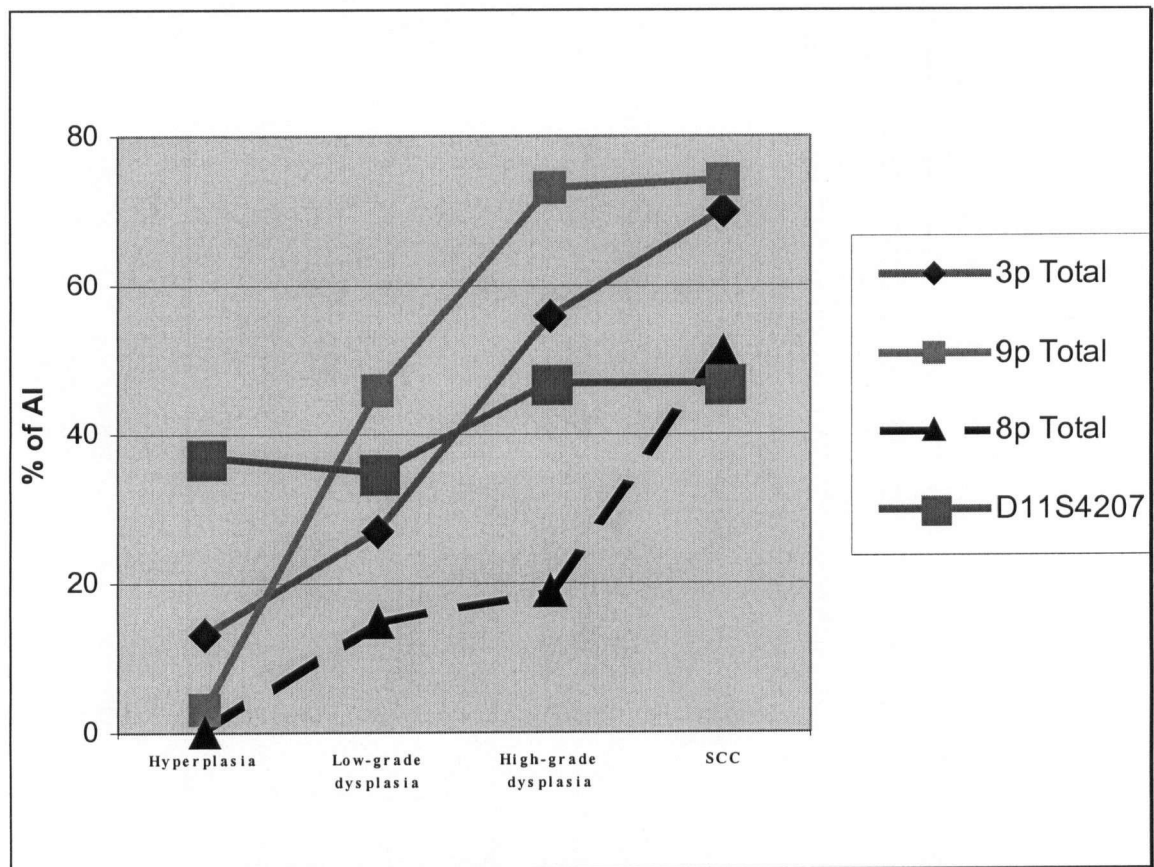
Microsatellite markers at 3p14 (*D3S1228, D3S1234, D3S1285 and D3S1300*), 9p21
(*INFA, D9S1751, D9S171* and *D9S1748*) and 8p (*D8S261, D8S262* and *D8S264*).

VI.3. The timing of induction of AI at *D11S4207*, *int2* and *D11S1778* during histological progression.

In this study, the 143 cases of primary oral premalignant lesions studied in the previous section for *D11S4207* were further assayed using the markers *int2* and *D11S1778*. The purposes were two folded, the first was to determine whether or not the alteration at *D11S4207* was distinct from that occurring at these 2 loci that we have previously studied in this laboratory (see section I.2.3.f.) with a small number of cases, and the second was to determine the temporal changes of these two loci in different stages of oral premalignant and malignant lesions. The data suggest that the stage in which alterations occur in these 3 regions is different for *D11S4207* compared with the other 2 regions (Table 10)

In contrast to the results obtained with *D11S4207*, AI was rarely observed in hyperplasia and low-grade dysplasia for the markers *int2* and *D11S1778*. AI was present at *D11S1778* in only 1 of 27 (4%) hyperplastic lesions and 3 of 51 (6%) low-grade dysplasias; for *int2* theses frequencies were 2 of 23 lesions (9%) and 2 of 36 (6%) respectively. For both *int2* and *D11S1778*, there was a significant increase in AI in high-grade dysplasias, with the alteration occurring in 9 of 42 (19%) lesions for *D11S1778* (P = 0.0325) and in 12 of 41 (29%) lesions for *int2* (P = 0.0081). Further increases in AI were noted with invasion for both *D11S1778* and *int2*, with AI being present in 24 of 73

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(33%) SCCs and 29 of 62 (47%) SCCs, respectively. However, these increases in AI between high-grade dysplasias and SCC were not significant (Table 10).

Allelic imbalance data for these three loci were also plotted in Figure 10. This figure clearly shows the low AI frequencies in early lesions and the sharp rise of these frequencies from low-grade dysplasia to high-grade dysplasia, and then to invasive SCCs for both *D11S1778* and *int2*. In contrast, *D11S4207* displays high AI in the earliest lesions and only a slight elevation of these frequencies between low-grade and high-grade dysplasia.

When AI frequencies are compared in SCCs for the 3 primers, there is no significant difference suggesting that at this stage a similar frequency of alteration is present in all 3 loci. In contrast, AI frequencies at *D11S4207* were significantly higher than those at either *D11S1778* or *int2* for all earlier stages: hyperplasia, low-grade dysplasia, and high-grade dysplasia (Table 10).

Figure 10. Comparison of AI frequencies observed at *D11S4207* with those seen with microsatellite markers at *D11S1778* and i*nt2*
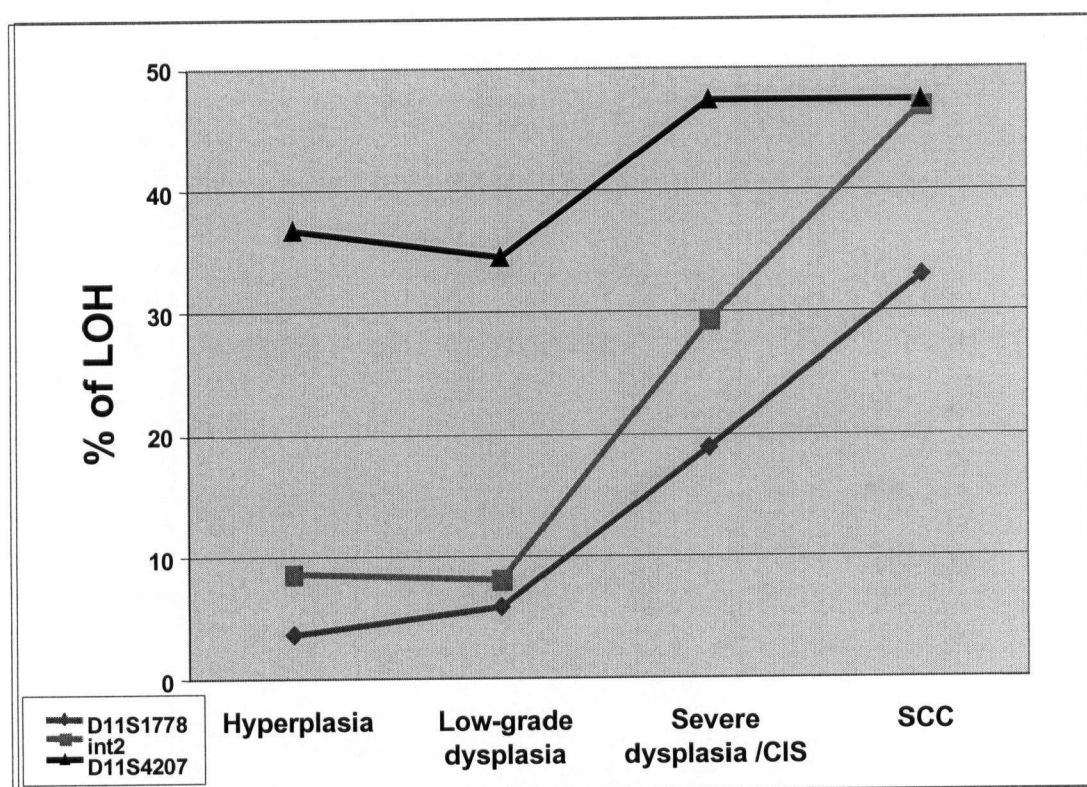
Table 10.   Allelic imbalance at *D11S4207*, *int2* and *D11S1778* oral premalignant lesions and SCC

Locus	Hyperplasia (n = 33)	Low-grade dysplasia (n = 54)	P value	High-grade dysplasia (n = 56)	P value (low- vs. high-grade dysplasia)	SCC (n = 91)	P value (SCC vs. high-grade dysplasia)
D11S1778	1/27 (4)[a]	3/51 (6)	1	9/42 (19)	**0.0325**[b]	24/73 (33)	0.2077
int2	2/23 (9)	2/36 (6)	1	12/41 (29)	**0.0081**	29/62 (47)	0.1003
D11S4207	7/19 (37)	10/29 (34.5)	1	18/38 (47)	0.3265	35/74 (47)	1

[a] Value given as number of samples showing loss/total number of informative cases (% of cases in parentheses)

[b] Bold means p< 0.05, was considered significant.

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VI.4. Further evidence in support of the AI at *D11S4207* being an independent event

Another way of demonstrating that the AI at *D11S4207* is occurring independent of alteration at the other 2 regions is to determine how frequently these alterations occur together or independent of each other in the same samples. Table 11 compares patterns of AI for *D11S4207* and *int2*. Table 12 provides similar comparisons for *D11S4207* and *D11S1778*.

As shown in Table11, in the majority of samples in which AI occurred at either *D11S4207* or *int2*, the alteration in these 2 regions was not 'synchronous', that is, the alteration occurred in only 1 of the 2 primers and not both at the same time. This was true for 75% of hyperplasias, 100% of low-grade dysplasias73% of high-grade dysplasias, and 56% of SCCs.

Table 11. Patterns of alteration at *D11S4207* and *int2*: frequencies at which
these alterations occur together or independent of each other

	# of cases informative at both loci	AI at *D11S4207* only	AI at *int2* only	AI at both *D11S4207* & *int2*	Total # cases with different pattern (%)[a]
Hyperplasia	13	2	1	1	3/4 (75%)
Low-grade dysplasia	22	5	1	0	6/6 (100%)
High-grade dysplasia	28	7	4	4	11/15 (73%)
SCC	51	9	9	14	18/32 (56%)

[a] Value given as number of samples showing AI for one primer but retention for the other
(% of cases in parentheses).

Table 12 gives similar data for *D11S4207* and *D11S1778*. Again, the alteration to these 2

regions was most often not synchronous. The alteration occurred in only 1 of the 2

regions in 100% of hyperplasias, 80% of low-grade dysplasias, 60% of high-grade

dysplasias and 70% of SCCs.

Table 12. Patterns of alteration at *D11S4207* and *D11S1778*: frequencies at which these alterations occur together or independent of each other

	# of cases informative at both loci	AI at *D11S4207* only	AI at *D11S1778* only	AI at both *D11S4207* & *D11S1778*	Total # cases with different pattern (%)[a]
Hyperplasia	15	5	0	0	5/5 (100%)
Low-grade dysplasia	29	8	0	2	8/10 (80%)
High-grade dysplasia	32	8	1	6	9/15 (60%)
SCC	63	18	8	11	26/37 (70%)

[a] Value given as number of samples showing AI for one primer but retention for the other (% of cases in parentheses).

VI.5. Fine-mapping at *D11S4207*

The next logical step in this research was to fine-map the region around *D11S4207* (using further primers) in order to determine the smallest region of AI. That region would then be examined for putative oncogenes/TSGs. An attempt was made to do this early in the development of the thesis. Two primers were selected for study that originally mapped to

either side of *D11S4207*: *D11S916* and *D11S4119*. The initial data suggested that we had successfully established telomeric and centromeric boundaries for this region of alteration. Unfortunately, a month ago, the location of these markers changed. The December 22,2001 Human Genome project draft shown in the University of California Santa Cruz database (http://genome.cse.ucsc.edu/index.html) now places the markers as shown in Figure 6.

Future studies will require the careful mapping of primers onto tiled BAC arrays, a more stringent way of localizing markers. However, at present our data suggests that there may be a telomeric boundary at *D11S4119* which is located approximately 0.5 megabase pairs from *D11S4207*. The data supporting this boundary is still weak with only 4 cases of SCC (161T, 414T, 448T and 529T) showing AI at *D11S4207* and retention at *D11S4119*.

VI.6. Allelic imbalance at chromosome 11q and malignant progression risk

An important question that must be answered with each marker that is proposed to be associated with progression is whether or not the presence of this alteration increases the risk of a premalignant lesion to transform into an invasive SCC.

In order to answer this question, I compared AI frequencies in early premalignant lesions

with known outcome. Prior data on these samples has been presented in a recent published paper (Rosin *et al.* 2000) and is described below. A decision was made to look for associations of *int2* and *D11S1778* to progression risk. *D11S4207* was not studied because it requires further mapping to better localize the region of alteration prior to using the primers on these very precious samples.

The lesions consist of two groups of hyperplasia and oral low-grade dysplasia. One group (n = 87) is from patients with no subsequent history of head and neck cancer. All but three of these cases had at least 3 years of follow up time. We refer to these cases as 'non-progressing'. The other group (n = 29) is from patients that later progressed to *CIS* or SCC at the same anatomical site. The interval between the primary lesions and later *CIS* or SCC had to be longer than 6 months. We refer to them as 'progressing'.

As shown in Table 13, there was no significant difference between the progressing low-grade dysplasias and those without a history of progressing in terms of *gender* (56% male in progressing cases vs. 57% of those without a history), *age* (mean age 58 years in progressing cases versus 55 in those without a history), *site, and smoking history* (of those with known habits, 78% of progressing cases vs. 85% of those without a history were smokers). However, on average, non-progressing cases were monitored for over twice the duration (96 versus 37 months) to ensure that progression did not occur. This lengthy follow-up time is to ensure that progression did not occur.

Table 13.   Demographic information of patients with low-grade dysplasia

Features	Non-progressing (n=54)	Progressing (n=23)	P-value
Age (mean, years)	55	58	0.416
Sex (% male)	57	56	1
% with smoking history	85	78	0.170
Follow-up (mean, months)	96	37	0.0001

Table 14 shows the association of AI at *D11S1778* and *int2* with progression. For non-progressing low-grade lesions, AI was noted in only 4/78 (5%) at *D11S1778* and 4/59 (7%) at *int2*. In contrast, AI was present 9/23 (39%) at *D11S1778* and 6/21 (29%) at *int2* in those morphologically similar but progressing lesions. The difference between progressing and non-progressing low-grade lesions is of statistical significance both at *D11S1778* ($p = 0.0002$), and at *int2* ($p = 0.0175$).

Table 14. Allelic imbalance of *D11S1778 (ATM)* and *int2 (int2-cyclin D1)* in progressing and non-progressing hyperplasia and low-grade dysplasia

	Low-grade lesions		
	Non-progressing (n=87)	Progressing (n=29)	*p-value*
D11S1778	4/78 (5) [a]	9/23 (39)	**0.0002** [b]
Int2	4/59 (7)	6/21 (29)	**0.0175**

[a] Value given as number of samples showing loss/total number of informative cases (% of cases in parentheses).
[b] Bold means p< 0.05, was considered significant.

The data were also examined for association with disease progression by using the Kaplan-Meier method. Time-to-progression curves were plotted as a function of AI at *D11S1778* or at *int2* (figure 11). A significant difference was observed for each of the loci. These data suggest that *D11S1778* and *int2* both mark regions in the DNA with genes that are associated with risk of progression to cancer.

Figure 11.   Probability of having no progression to cancer, according to AI at

D11S1778 **or at** *int2*
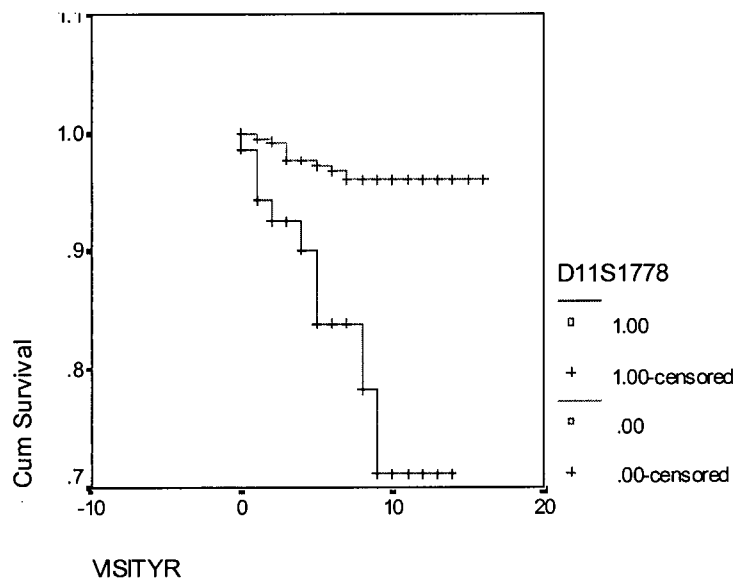
P = 0.0032
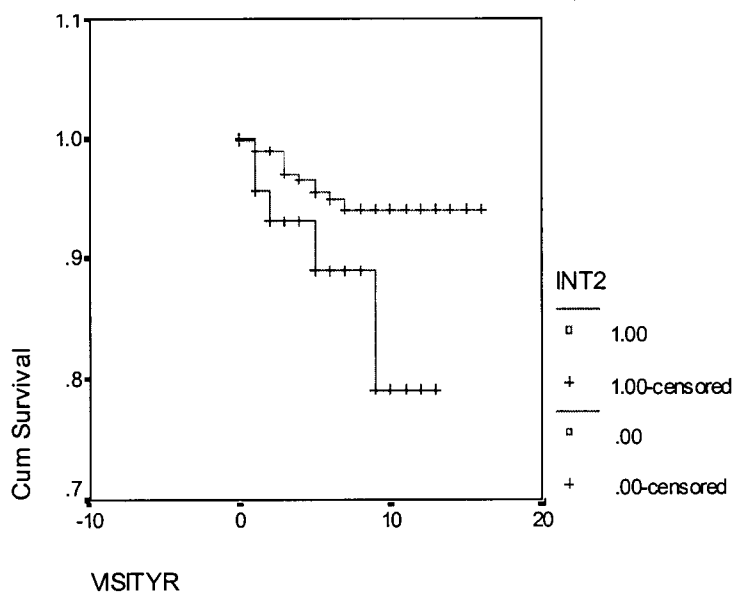
P = 0.0054

VII. DISCUSSION

To the best of my knowledge, this thesis has for the first time investigated a large number of premalignant lesions for genetic alterations at 11q. Three loci (*D11S4207, D11S1778* and *int2*) at 11q have been studied. *D11S4207* is a hot locus newly identified by this thesis; whereas *D11S1778* and *int2* are two loci that are widely studied in cancer but only limited information is available regarding their occurrences in oral premalignant lesions. For a better flow of the discussion, I will first discuss the temporal changes of the 3 loci during the multistage oral carcinogenesis, and then discuss the relationship between cancer progression of oral premalignant lesions and 11q changes, and finally the new locus, *D11S4207,* as a potential new hot spot containing tumor gene(s).

VII.1. Allelic imbalance of genes at *D11S4207, D11S1778* and *int2-cycline D1* during multistage oral carcinogenesis

The histological progression model for head and neck SCC is well established. There are strong evidences indicating that accumulations of changes to critical control genes (oncogenes and TSGs) underline the progression of lesions from hyperplasia to increasing degree of dysplasia (mild, moderate, severe), and to *CIS* and finally to invasive SCC. Currently a number of tumor genes and even more potential chromosome loci containing tumor genes have been established in oral SCC but information on these genes

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and loci in the early stage of oral lesions are limited in number and scope due to the difficulty of obtaining suitable specimens for analysis and to technical problems associated with working with very small lesions and minute amounts of DNA (Zhang *et al.*, 1997; Califano *et al.*, 1996; Roz *et al.*, 1996; Mao *et al.*, 1996b; Emilion *et al.*, 1996). Although a molecular progression model has been first proposed by Califano *et al.* (1996), and later refined by Rosin *et al.* (2000), genes and chromosome loci investigated in the model are limited. Understanding of the additional genetic changes and of their timing during the molecular progression of oral cancer is critical for our further understanding of the mechanisms of the tumor progression and prediction of cancer risk of oral premalignant lesions as well as intervention and management of high-risk oral lesions.

This thesis has studied a large number of oral premalignant lesions at different stages of progression for alterations for chromosome 11q at *D11S4207, D11S1778* (11q22-23) and *int2* (*int2-cyclin D1* region) to determine the timing of the alterations at these loci.

VII.1.1.   Temporal changes of the 3 loci at 11q

This thesis provides a great deal of genetic information, for the first time, of timing and frequency of AI at these three loci in oral carcinogenesis, which is essential to help better understanding of the mechanism of oral carcinogenesis and the role of 11q.

Temporally, allelic imbalance at *D11S4207* (the new gene site) is markedly different from those at *D11S1778* (11q22-23) and *int2* (*int2-cyclin D1* region). AI for *D11S4207* (the new gene site) occurred very early during the multistage carcinogenesis with only slight increase in the frequency of AI with progression of the oral lesions from low-grade to high-grade lesions and finally to invasive SCC. In oral hyperplasia, 7/19 (37%) demonstrate allelic imbalance for the new gene site. This rate is much higher than that seen for 3p and 9p losses in oral hyperplasia, both of which have been shown to occur early in the development of oral and many other solid cancers (Rosin *et al.*, 2000). However, unlike the changes seen for 3p and 9p losses, which steadily increase with progression of oral lesions, the rate of allelic imbalance seen in oral hyperplasia at *D11S4207* (the new gene site) has not markedly increased with progression of the oral lesions. Even when the rate of the allelic imbalance of the invasive oral SCC (35/74, 47%) is compared with that of the oral hyperplasia (7/19. 37%), the result is far from significantly different ($P = 0.4506$).

Unlike the new gene locus, allelic imbalances at *D11S1778* (11q22-23) and *int2* (*int2-cyclin D1* region) are rare at low-risk oral premalignant lesions. Both of these markers show markedly increased allelic imbalance with advent of high-grade dysplasias, which contain significantly increased alterations at these markers compared to low-grade dysplasia and hyperplasia ($P = 0.0352$ for *D11S1778* and $P = 0.0223$ for *int2*). The rise in the frequency of allelic imbalance continued in the invasive SCCs.

VII.1.2.   Significance of the changes of the 3 loci at 11q

Studies of temporal changes of allelic imbalance during multistage oral carcinogenesis

can provide critical information on the role of a presumptive tumor gene in the

development of oral cancer even prior to the identification of the actual tumor gene. For

example, if a gene were mainly altered during the transformation of preinvasive lesions to

invasive lesions, it would suggest that the gene plays a role in the tumor invasion.

VII.1.2.1. Significance of AI at D11S4207

The finding of a high frequency of AI at *D11S4207* (the new gene site) in oral lesions

with very low cancer risk (37% of hyperplasia) and the finding of a similar frequency or

only slightly higher frequency of AI in oral lesions with higher cancer risk (34.5% low-

grade dysplasia and 47% of high-grade dysplasia) or even in SCC (47%) are unusual.

Our previous studies on other chromosome regions in oral lesions generally demonstrate

a significant rise in the losses at a specific stage(s) of oral cancer development. For

example, losses at 3p and 9p were found to be significantly (or approaching significantly)

increased from hyperplasia to low-grade dysplasia, and then continued to be markedly

increased at later stages (high-grade dysplasia or SCC). On the other hand, a significant

increase in LOH at 17p occurred with the formation of high-grade dysplasias, whereas

significant increases in LOH at 8p and 13q occurred with the advent of invasive SCC (see

Table 1 at Section I.2.3.f).

Is such early occurrence of a high frequency of AI at *D11S4207* without obvious increase in the frequency of AI with progression of oral lesions exceptional? In a recent study, we have noticed similar temporal changes of AI in another chromosome region with presumptive tumor genes during oral carcinogenesis. AI at 14q31-32 was shown to be high in low-grade dysplasia (17/51, 33%), but the frequency of AI did not increase with progression of oral lesions: AI was noted in 10/23 (30%) of high-grade dysplasias and 12/33 (36%) invasive SCCs (unpublished data from this lab).

The significance of such early occurrence of AI without accompanying increase with progression of oral lesions remains speculative. Since demonstration of AI by microsatellite analysis is an evidence of clonal expansion of cells with growth advantages (required by tumorigenesis), the presence of AI, even in hyperplasia, could not be ignored. The explanation for lack of further increase in the frequency of AI could be complicated, including alternate modes of inactivation/alteration such as epigenetic silencing of gene expression by promoter methylation (Thiagalingam *et al.*, 2002), and different cancer development pathways, or simply because only one primer, *D11S4207*, has been used to probe the region, and an increased number of primers for the region may identify further cases with the alteration.

Whatever function or significance the new locus has, it is clear that AI at *D11S4207* occurred independently of AI at *int2* (*D11S4207* is localized at a region 20 Mb telomeric to *CCND1-int2* region), and of AI at *D11S1778* (11q22-23). The results in Section VI.4

showed that the majority of samples did not show AI at *D11S4207* simultaneously or synchronously with either AI at *int2* or at *D11S1778* (see Tables 11 and 12). At *int2*, the discordance with *D11S4207* occurred in 50% of hyperplasias, 100% of low-grade dysplasias, 73% of high-grade dysplasias, and 56% of SCCs. At *D11S1778*, the discordance *D11S4207* occurred in 100% of hyperplasias, 80% of low-grade dysplasias, 60% of high-grade dysplasias and 70% of SCCs. The rates should be even higher given the fact that the assay does not yield any information on whether or not the maternal or paternal allele is being altered in samples showing AI. Thus, estimates on the actual percentage of cases in which 2 events are giving rise to alterations at these 2 loci are even lower. An alteration could occur on the maternal allele of one locus and on the paternal allele of another loci and still be recorded as a single event.

Several studies have suggested the existence of fragile sites at 11q13 region (Conquelle *et al.*, 1997; Jin *et al.*, 1998; Shuster *et al.*, 2000), including the region harboring *D11S4207* (Jin *et al.*, 1998). One speculation for the early occurrence of AI at *D11S4207* is that cells with such fragile site alterations have an increased genomic instability, which subjects the cells with the AI to further genetic changes. In our recent publication, it has been shown that AI at 3p &/or 9p occurred early and were found in almost all oral premalignant lesions that later progressed into cancer, suggesting AI at these loci are essential for cancer formation. Table 15 examines the relationship between AI at *D11S4207* and AI at 3p &/or 9p, the proposed essential changes for oral cancer development, to determine the potential importance of AI at *D11S4207*.

Of the 29 cases of low-grade dysplasia, 10 cases have AI at *D11S4207*, whereas 19 have

no alteration at the site. AI at 3p &/or 9p was noted in a significantly higher proportion

of lesions with AI at *D11S4207* as compared to lesions without AI at *D11S4207*: 9 (90%)

of 10 lesions vs. 7 (37%) of 19 cases ($P = 0.0084$). Such results suggest an association

between genetic alterations at *D11S4207* and those at 3p and 9p.

Table 15. **Association of allelic imbalance at *D11S4207* and 3p &/or 9p in low-grade dysplasias**

		3p and/or 9p		*P-value*
		AI	**Retention**	
D11S4207	**AI**	9	1	*0.0084*
	Retention	7	12	

VII.1.2.2. Significance of AI at D11S1778 (11q22-23) and int2 (int2-cyclin D1 region)

Although AI at 11q13-22 has been extensively investigated in head and neck cancer, this

is the first study to investigate *int2-cyclin D1* genes at 11q13 and 11q22-23 region

separately in oral premalignant lesions with a large number of oral lesions at different

stages of cancer progression. AI at the two loci was rare in hyperplasia and low-grade

dysplasia. Both *D11S1778* (11q22-23) and *int2* (*int2-cyclin D1* region) showed

significant increase in the frequency of AI   with formation of high-grade oral

dysplastic lesions, and the increase continued with formation of SCC. Such results indicate that lesions with these changes have high-risk for cancer progression, and also support the literature that genes at 11q13-22 play important roles in tumor instability and invasion.

VII.2.   Allelic imbalance at *D11S1778* and *int2* is associated with cancer risk.

A salient advantage for microsatellite analysis is that data from microsatellite analysis not only could provide clue for the identification of target genes, but also could be used as markers for diagnosis and prognosis, even prior to identification of the actual target genes. The latter is particularly important as from a clinical point of view; the ultimate significance of molecular studies is that molecular markers can be used to guide clinical management, including prediction of risk of cancer progression of oral premalignant lesions.

Despite of the obvious significance and importance of linking molecular results with clinical outcome, few such studies are available for premalignant lesions. Genetic studies of oral premalignant lesions are difficult even without the requirement of the clinical outcome. In this study, microdissected early oral premalignant lesions from 116 patients with or without a history of progression into CIS or invasive SCC were analyzed for AI at *D11S1778* and *int2* in order to determine the potential roles of these gene loci in the

progression of low-grade oral premalignant lesions.

As discussed above, the results of one of my studies show that allelic imbalances at *D11S1778* and *int2* occur mainly in high-grade oral dysplastic lesions and invasive oral SCCs. These results would suggest that these molecular markers could serve as risk markers for cancer progression since high-grade dysplasias are known to have a high-risk for cancer progression.

Low-grade lesions (those without dysplasia or with low-grade dysplasia) were chosen for this study because the majority of these lesions will not progress into cancer. Currently it is not possible to identify the small percentage of progressing low-grade lesions from the majority of morphologically similar but non-progressing lesions. Since AI at *D11S1778* and *int2* were rare in the low-grade lesions in my study, in this study we asked whether AI at *D11S1778* and *int2* indicate a risk of cancer progression, and whether those low-grade dysplasias with AI at the two loci had increased cancer risk.

The study showed that non-progressing low-grade lesions (without dysplasia or with low-grade dysplasia) had significantly lower frequencies of allelic imbalance at both *D11S1778* and *int2*, as compared to the non-progressing hyperplasia (Table 14). For non-progressing low-grade lesions, AI was noted in only 4/78 (5%) at *D11S1778* and 4/59 (7%) at *int2*, significantly lower than those morphologically similar but progressing lesions: 9/23 (39%) at *D11S1778* ($P = 0.0002$) and 6/21 (29%) at *int2* ($P = 0.0175$). Such study results lend strong support to the hypothesis that AI at both *D11S1778*

(11q22-23) and *int2* (*int2-cyclin D1* region) is associated with high cancer risk for oral premalignant lesions and that morphologically low-grade lesion with AI at either of the two loci may indicate high cancer risk despite of a low-risk morphology. This thesis provide solid evidence that AI at *D11S1778* and *int2* could be used as potential markers to identify high-risk lesions at early stage, which may have important impact on the clinical diagnosis and management.

VII.3.*D11S4207*, a new hot spot at 11q13

As mentioned in literature review, despite of discovery of a number of genes at the 11q13 region, it is generally believed that many more have yet to be identified from the site, which is a major region that plays a key role in controlling chromosomal instability and progression of tumors (Bekri *et al.,* 1997, Izzo *et al.,* 1998, 1999; Zhou *et al.,* 1996; Gebhart *et al.,* 1998). Identification and localization of these potential genes will contribute to the understanding of the roles of 11q13 region in tumorigenesis, including oral carcinogenesis.

This thesis has identified a new hot spot, *D11S4207*, for tumor gene at 11q13 region. Of the 91 oral SCC investigated, 74 were informative and 35/74 (47%) demonstrated allelic imbalance at *D11S4207*.

Subsequent to the discovery of the new locus, I investigated the boundaries of the region

of alteration. My data had indicated successful establishment of telomeric and centromeric boundaries for the region of alteration with microsatellite markers *D11S916* and *D11S4119*, until one month ago when the location of these markers changed (http://gonome.cse.ucsc.edu/index.html), although two cases of SCC did suggest that there may be a telomeric boundary at *D11S4119*, which is located approximately 0.5 megabase pairs from *D11S4207*.

Despite of the need for further fine mapping of the region, existing data do support that this is a new hot spot containing tumor gene(s). Not only there is a high frequency of AI at *D11S4207* in oral SCCs, a high frequency of AI at *D11S4207* was also noted in preinvasive lesions including low risk oral hyperplasias. Cancers are characterized by an intrinsic genetic instability that frequently results in a cascade of nonspecific genetic alterations, which make the identification of alterations to critical control genes difficult. Consequently it could be argued that AI at *D11S4207* in oral SCCs could be non-specific, even though the frequency of AI (47%) is too high for that argument. The presence of AI at the same region in preinvasive lesions, however, strongly support that the alteration is not random 'gun-shot' effects, but rather true hot spot containing tumor gene(s).

The identification of this new hot spot and further fine mapping of the region will lead to pinpoint the location of putative tumor genes for further analysis and is critical for further sequencing to identify new genes and related functions, and add further understanding to the role of 11q13 in the cancer development.

VII.4. Ending Mark

Although my study identified a new locus and also provides AI frequencies of three loci at 11q: *D11S4207*, *int2* and *D11S1778* in oral cancer and premalignant lesions, there are some limitations as well. One major limitation is that there was only one primer used for each locus of the three loci at 11q. This is insufficient since each of the loci probably contains multiple genes; hence the data provide only information of 'global' changes on the region. The use of only one primer for each locus also increases the percentages of cases studied being non-informative, which lowers the sample size with data and increases the possibility of Type II error in statistical analysis. For example, 14 (42%) out of 33 of hyperplasia cases were non-informative at *D11S4207*.

There are many exciting things I would like to continue on the project in the future. This will include fine map the locus of *D11S4207* to localize and ultimately sequence and identify the candidate tumor genes in this region. Currently it seems that careful mapping of primers onto tiled BAC arrays would be the choice of techniques although other newer techniques could be out very soon with the rapid advances in technologies. The identification of the gene(s) could be followed by immunohistochemical studies of the gene products to investigate the protein changes of the genes. I would also like to employ other techniques, such as FISH to determine whether the alteration at the new locus is deletion or amplification. The information will be important for determination whether the genes are tumor suppressor genes (deletion) or oncogenes (amplification) and further mechanistic studies.

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