THE USE OF MICROSATELLITE MARKERS TO ASSESS THE MALIGNANT POTENTIAL OF ORAL PRECANCEROUS LESIONS AND ORAL LICHEN PLANUS

by

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B.M.L.Sc., University of British Columbia, 1991

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the School

of

Kinesiology

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SIMON FRASER UNIVERSITY

September 1997

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0-612-24207-2

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ABSTRACT

Many, if not all, oral squamous cell carcinomas (SCC) are believed to develop through sequentia stages involving progressing degrees of dysplasia (premalignant lesion) and finally invasive SCC. It is now generally accepted that underlying this multistage process are a series of genetic change in critical genes called oncogenes and tumour suppressor genes (TSG). Study of the sequence o these genetic changes in premalignant lesions is particularly important since such information is essential for early diagnosis and intervention.

Tumourigenesis occurs after loss of loci containing TSGs on specific chromosomes. Recently, powerful new procedures have been developed to detect this loss, even from archival material. One technique, microsatellite analysis, detects loss of heterozygosity (LOH). LOH has been found to be a common event in many head and neck cancers, particularly when it occurs on chromosomes 3p, 9p, and 17p. However, information on LOH in oral premalignant lesions (oral dysplasias) is sparse.

This thesis used microsatellite analysis to examine allelic loss in oral dysplasias. In addition, oral lichen planus (OLP) was included in the analysis; whether or not OLP is a premalignant lesion is subject of much current debate. Sixty one cases of dysplasias and 33 cases of OLP were assessed for allelic loss at 9 loci located on chromosomes 3p, 9p, and 17p. In addition, 29 cases of benign reactive lesions and 28 SCCs were analyzed and used as negative and positive controls respectively.

The study has, for the first time, shown that with progression of oral dysplasias there is progressive development of LOH. LOH on any of the three arms was present in only 14% of reactive lesions, but increased with progression of the oral lesions. It was present in 43% of mild dysplasias, 57% of moderate dysplasias, 79% of severe dysplasia/c.i.s. and 100% of SCC. The frequency of this loss correlated significantly with progression of oral lesions ($p = 0.0167$).

Furthermore, LOH was detected on only a single arm in reactive lesions but occurred on more than one chromosome in dysplasia and cancer. The frequency of this multiple loss (multiple hit) again correlated significantly with progression of oral lesions ($p = 0.0167$).

Only 6% of OLP showed LOH, a frequency that is even lower than that of the benign reactive lesions. Similar to reactive lesions, the allelic loss noted in OLP was again only present on single chromosome arms. While these findings do not support OLP as a lesion at risk for malignant transformation, such results need to be confirmed by use of other genetic markers since OLP may undergo malignant transformation through genetic pathways different from those of oral dysplasia.

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ABBREVIATIONS

c.i.s.:	carcinoma *in situ*
HNSCC:	squamous cell carcinoma of the head and neck
LP:	lichen planus
LOH:	loss of heterozygosity
OLP:	oral lichen planus
PCR:	polymerase chain reaction
SCC:	squamous cell carcinoma
TSG:	tumour suppressor gene
WHO:	World Health Organization

1. OVERVIEW

Head and neck squamous cell carcinoma (HNSCC) is responsible for 5% of malignancies in the Western hemisphere (Vokes *et al* 1993), but the rate of HNSCC is as high as 40% in the Far East and India (Saranath *et al* 1993). Patients with HNSCC and oral cancer tend to have a high rate of local recurrence and of second primary tumours throughout the aerodigestive tract even after successful treatment of the first tumour. This phenomenon, termed field cancerization, was first noted by Slaughter *et al* (1953) when he described carcinogen-induced changes throughout the upper aerodigestive tract resulting in multiple primary malignancies.

Despite the advances made in cancer treatment, five year survival rate for most cancers, with the exception of some hematological malignancies, has not improved over the last several decades. Early diagnosis and treatment still offers the best chance of cure for cancers. This has resulted in a recent emphasis on the study of early phases of carcinogenesis (premalignant lesions), and an increased awareness of the importance of screening and management of the carcinogenesis process at early stages. According to the World Health Organization (1978), premalignant lesions are morphologically altered areas of a tissue in which cancer is more likely to occur than in its apparently normal counterpart. Only a small percentage of premalignant lesions progress into cancer (Banoczy and Sugar 1972; WHO, 1978).

In the oral cavity, premalignant lesions most often present clinically as leukoplakia, and occasionally as erythroplakia. As with other premalignant lesions, the majority of oral premalignant lesions do not become malignant. The malignant potential of oral lesions are currently judged solely on the degree of dysplasia by microscopic examination of tissue sections stained with hematoxylin and eosin and/or other histolochemical procedures (WHO, 1978;

Beckstead *et al* 1996). According to the severity of dysplasia present in a lesion, oral premalignant lesions can be classified histologically into mild dysplasia, moderate dysplasia, sever dysplasia, and carcinoma *in situ* (c.i.s.) before becoming invasive squamous cell carcinoma (SCC (histological tumour progression model). While the histological progression model offers a good predictive value for late preinvasive lesions (severe dysplasia and c.i.s.), this model is poor in predicting the malignant potential of early premalignant lesions (mild and moderate dysplasia). Biomarkers that will help in identifying premalignant lesions that have higher malignant potential are highly needed.

Lichen planus (LP) is a common mucocutaneous inflammatory disorder of uncertain etiology that occurs frequently in the general population (estimated prevalence of 1%). It commonly affects the oral cavity and has been regarded to be a premalignant oral lesion (Scully & El-Kom 1985; Axell 1976; Pindborg *et al* 1972). However, there is controversy regarding the premalignant potential of oral lichen planus (OLP). Some argue that only those OLPs that have developed dysplastic changes have a malignant potential, whereas OLP *per se* is not premalignant. Studies are needed to resolve the controversy.

With rapid development of molecular biology techniques, there is increasing evidence that multistage carcinogenesis is underlined by a series of genetic changes in oncogenes and tumour suppressor genes (TSG), which must be lost for tumourigenesis to occur. Study of the sequence of these genetic changes in premalignant lesions is particularly important. The understanding of multistage carcinogenesis at early stages is critical to early diagnosis and intervention of the disease process.

This thesis will employ microsatellite analysis, a powerful new procedure that detects loss of heterozygosity (LOH), to study the loss of TSGs in oral dysplastic lesions and OLP. LOH has been found to be a common event in many head and neck cancers, particularly those occurring on chromosomes 3p, 9p, and 17p (Califano *et al* 1996; El-Naggar *et al* 1995; Emilion *et al* 1996; Mao *et al* 1996; Roz *et al* 1996). However, information on LOH in different stages of oral premalignant lesions (oral dysplasias) and OLP is lacking.

Oral cancer is generally believed to develop through sequential stages of premalignant lesions: mild, moderate, severe dysplasia, and c.i.s. (WHO, 1978). The following section will first review oral premalignant and malignant lesions, including the controversial lesion OLP. It will then briefly summarize the genetic changes, mainly allelic losses at chromosome 3p, 9p, and 17p in head and neck premalignant and malignant lesions.

2. ORAL PREMALIGNANT AND MALIGNANT LESIONS

2.1. Oral Mucosa

The mucosa that lines the oral cavity consists of overlying epithelium and underlying connective tissue, similar to skin. The majority of carcinomas of the oral cavity arise from the lining epithelium of oral mucosa. Normal oral lining epithelium is composed of stratified squamous epithelium, which consists primarily of basal cells and prickle cells. The cuboidal-shaped basal cells form the first layer on the basement membrane that separates the epithelial lining from the underlying connective tissue layer, the lamina propria. Basal cells have dividing capacity and give rise to the prickle layer located above them.

When a basal cell divides, it may give rise to new basal cells or differentiate to form the larger polyhedral shaped prickle cells. As the prickle cells mature, they push towards the surface where they shrink in size, become long and flat, and are eventually desquamated. Oral epithelium is usually non-keratinized except for mucosae lining of the attached gingiva, hard palate, dorsal surface of the tongue, and lips.

The connective tissue or lamina propria consists of loose connective tissue containing blood vessels, lymphatics, and fibroblasts, as well as collagen and elastic fibers. It functions to nourish and support the epithelial layer.

2.2. Etiology of Oral Cancer

Several factors are involved in the pathogenesis of SCC of the oral cavity and can be used to identify those at risk. The main risk factors of oropharyngeal carcinogenesis are tobacco usage and/or alcohol consumption.

Tobacco usage is the most important etiological factor for HNSCCs (Bundgaard *et al* 1995; Paterson *et al* 1996; Andre *et al* 1995). Both the smoking and the smokeless forms of tobacco have been strongly associated with development of HNSCC. It is well known that tobacco contains various carcinogens such as aromatic hydrocarbons and nitrosamines. These carcinogen effects of tobacco are dose and time dependent: more and longer use of tobacco products increases risk of developing cancer. The risk of cancer development is particularly high for individuals who began smoking before age 18 and sustained the habit for 35 years or longer.

Subjects who smoked non-filter cigarettes, who "rolled their own", or who "inhaled" all had a higher risk compared to controls (Andre *et al* 1995). Cessation of smoking has been found to lower risk of HNSCC as compared to those who continue to smoke, but only former casual smokers (<7 cigarettes/day) could get to the same low risk as non-smokers (Andre *et al* 1995).

The risk associated with alcohol consumption is not as well understood as tobacco. It is not clear whether alcohol by itself is carcinogenic, since heavy drinkers usually tend to smoke as well. Hence, it is difficult to separate the effects of alcohol on carcinogenesis. Some believe that alcohol only acts as a cocarcinogen. The possible mechanisms proposed for the cocarcinogenic effects of alcohol include the dehydrating and irritating effect it has on mucosa, or it's potential function as a solvent for carcinogens (Gerson, 1990). The cocarcinogenic effects may also result from liver damage by alcohol, which causes reduced clearance of carcinogens (Gerson, 1990). Hsu *et al* (1991) also suggested that ethanol may temporarily inhibit DNA repair. There is no doubt, however, that combined usage of tobacco products and alcohol causes markedly increases the risk of cancer development.

The roles these etiological factors play in the incidence of HNSCC is strongly supported by the distribution of oral cancers. HNSCC is responsible for 5% of malignancies of the Western hemisphere (Vokes *et al* 1993) and is caused mainly by smoking. On the other hand, the rate of HNSCC is as high as 40% in the Far East and India (Saranath *et al* 1993), where not only smoking is popular but also usage of smokeless tobacco, starting from a young age. Smoking of bedi (tobacco rolled in a temburni leaf) and reverse-smoking are also prevalent in these regions. These subjects often also have dietary deficiencies such as vitamins A and C, iron and other trace

elements (Paterson *et al* 1996). While nutritional deficiency is not carcinogenic, the deficiency may cause decreased protection against development of cancer.

Genetic predisposition and family history also contribute to risk of any type of cancer. In a Brazilian study of HNSCC, Foulkes *et al* (1995) found that a family history of any cancer significantly increased one's risk of developing HNSCC. This risk was especially high in first degree relatives, particularly among siblings. The relative risks calculated were adjusted for age, sex, and city of admission, as well as alcohol and tobacco use.

When discussing possible etiological factors of cancer of the aerodigestive tract, one must consider the theory of field cancerization. In 1953 Slaughter *et al* introduced the concept of "field cancerization" to explain the high incidence of second primary tumours arising in the aerodigestive tract. According to this theory, the carcinogen-exposed field, i.e., the aerodigestive tract exposed to heavy smoking, contains multiple lesions at different stages of cancer development reflecting the independent mutation of multiple cells across the exposed epithelium. This theory is generally used to explain why cancer patients have a high risk of developing a second primary tumour in the carcinogen exposed field. HNSCC patients not only have a high rate of local recurrence of their cancer but also have a 2-3% chance of developing a second primary, independent cancer each year (Berg *et al* 1970; Licciardello *et al* 1989).

Another theory used to explain the high rate of formation of second primary tumours in HNSCC patients is that a genetically changed single cell may spread to an adjacent mucosal surface. Thus, the second primary tumour is just a malignant clone of a genetically similar primary

tumour. Presently, the bulk of evidence support the field cancerization theory, although some new evidence supports the second hypothesis (Bedi *et al* 1996).

2.3. Oral Premalignant Lesions

A premalignant lesion is "a morphologically altered tissue in which cancer is more likely to occu than in its apparently normal counterpart", according to the World Health Organization (1978). Most oral premalignant lesions present clinically as leukoplakias, and occasionally as erythroplak (WHO, 1978). These terms are merely clinical descriptions and their risk for malignant transformations must be assessed histologically by the presence or absence of dysplasia and the degree of dysplasia.

Leukoplakia means "white patch" and occurs on mucous membranes such as the mucosa of the oropharynx, larynx, esophagus, and genital tract. Not necessarily white, leukoplakias also may appear yellow to light brown, especially in smokers. The World Health Organization (WHO) defines leukoplakia as a white patch which is not removed by rubbing and is not classifiable as an other oral disease. Leukoplakia is a clinical entity, and the term can be only used clinically.

Leukoplakias histologically demonstrate epithelial hyperkeratosis and/or acanthosis with or without dysplasia. The more severe the degree of dysplasia, the greater the potential for malignant transformation. Overall, 4% of leukoplakias eventually undergo malignant transformation (McFarland *et al* 1996). Lesions that have red granular areas and/or spreading margins, as well as fissured or thickened lesions, all carry increased risk for cancer (WHO, 1978)

Leukoplakias occur throughout the oral cavity, with those in the buccal and mandibular sites being the most common. Leukoplakias from the floor of the mouth, ventrolateral surface of the tongue, soft palate, and lips hold an increased risk for dysplasia and squamous cell carcinoma. Hence, these regions are called high-risk areas whereas the other oral sites are called low-risk areas (Banoczy and Sugar, 1972).

Erythroplakia, a lesion much less common than leukoplakia, presents clinically as a red patch. This lesion histologically shows atrophy of the epithelium and may have erosion of the epithelial layer, and it invariably shows either dysplasia, frequently severe in degree, or SCC (WHO, 1978) The World Health Organization defines erythroplakia as "a lesion of the oral mucosa that present as bright red, velvety plaques which cannot be characterized clinically or pathologically as being due to any other condition" (WHO, 1978). Common sites for erythroplakia include the floor of the mouth and the posterior two thirds of the tongue. The lesions may occasionally appear as white or yellow nodular bumps of keratinization on a red background.

The majority of oral premalignant lesions will not progress into cancer (WHO, 1978; Banoczy an Sugar 1972). To assess the risk of malignant transformation of leukoplakia or erythroplakia, a biopsy is taken of the clinical lesion to examine the presence of dysplasia and the degree of dysplasia. The term dysplasia refers to the classical cytological and histological changes in intraepithelial lesions that are historically associated with an increased risk for cancer (WHO, 1978).

The World Health Organization has established the following criteria for histological diagnosis of oral dysplasia (1978):

1. Loss of basal cell polarity
2. More than 1 layer of basaloid cells
3. Increased nuclear to cytoplasmic ratio
4. Drop-shaped rete ridges
5. Irregular stratification
6. Increased and abnormal mitoses
7. Mitotic figures in the superficial half of the epithelium
8. Cellular pleomorphism (variation in shape and size)
9. Nuclear hyperchromatism (dark staining nuclei)
10. Enlarged nucleoli
11. Reduction of cellular cohesion
12. Keratinization of single cells or cell groups in the spinous cell layer

Dysplastic lesions are further divided into mild, moderate, and severe forms depending upon how much of the tissue is dysplastic. Mild dysplasia are lesions in which the dysplastic cells are confined to the basal layer and the cells exhibit the smallest degree of the above changes. With moderate and severe dysplasia, the epithelial layers involved and the severity of the cellular changes are progressively increased. In carcinoma *in situ*, the dysplastic cells occupy the entire thickness of the epithelium (bottom to top changes) although the basement membrane is still intact. Invasion of dysplastic cells through the basement membrane into the underlying stroma and the dissemination of these cells to other sites through lymphoid and circulatory systems are events associated with development of invasive SCC (WHO, 1978).

2.4. Oral Lichen Planus

Lichen planus (LP) is a common mucocutaneous inflammatory disorder of uncertain etiology tha occurs frequently in the general population (estimated prevalence of 1%) and has been regarded by some to carry a potential elevation in cancer risk (Scully & El-Kom 1985; Axell 1976; Pindborg *et al* 1972). The disorder derives its name from the reticular, white, "lichen-like" lesioı that are present on the oral mucosa and/or skin of patients. These lesions are usually asymptomatic, although the occasional case has erosive areas causing discomfort. Oral lichen planus (OLP) occurs most often on the buccal mucosa, followed by the tongue, and gingiva. (McFarland *et al* 1996).

While the cutaneous form tends to resolve itself spontaneously, the oral form is much more persistent with remission being infrequent or absent. Several studies have observed OLP patients over time and have noted the development of SCC at the oral lesion site in a small percentage of patients. Rates of malignant transformation in these studies vary from study to study, averaging 1-2%. The results and references of a few studies are summarized in Table 1.

Table 1. Rate of Malignant Transformation in OLP

Author	Country of Origin	Observation Time (years)	No. of LP Cases	Rate of Malignant Transformation	Linked to Tobacco Use
Holmstrup *et al* (1988)	Denmark	10.1	611	1.5%	Unknown
Sigurgeirsson *et al* (1991)	Sweden	9.9	2071	0.4%	Unknown
Murti *et al* (1986)	India	5.1	722	0.4%	Yes
Salem (1989)	Saudi Arabia	3.2	72	5.6%	No

Despite the above information, opinions of dentists and oral pathologists tend to differ with respect to the malignant potential of these lesions. While some regard OLP as precancerous, others question whether OLP is a real premalignant lesion and have not followed them up regularly (Krutchkoff and Eisenberg, 1985; 1992).

It is not clear whether OLP is itself a risk lesion or whether an elevated cancer risk is only associated with those cases of OLP that contain dysplastic changes in them. Presence of dysplasia and degree of dysplasia is presently the most reliable parameter associated with precancerous lesions.

In 1985, the term "lichenoid dysplasia" was introduced by Krutchkoff and Eisenberg to classify OLP lesions with accompanying dysplasia. They hoped to demonstrate that OLP by itself is not premalignant and that only lichenoid lesions with accompanying dysplasia should be regarded as

premalignant lesions. After studying several published cases of OLP with malignant transformation, it was concluded that most supposed OLP cases demonstrating malignant transformation were in fact lichenoid dysplasia (Krutchkoff and Eisenberg, 1985). Further retrospective studies by Urbizo-Velez *et al* (1990) and DeJone *et al* (1984) reported dysplasia present in 11% and 25% (both n=100) of the OLP lesions respectively. It is obvious that morphological approaches alone are not sufficient to resolve this controversy. An alternate approach may be to use molecular techniques to follow genetic changes in the lesions and to use this data to differentiate between high-risk and low-risk lesions.

OLP shows distinctive histopathological changes. This disease is thought to be a T-cell mediated autoimmune disorder. There is extensive thickening of most layers of the epithelial layer, particularly the stratum corneum, granular layer, and stratum spinosum. The latter layer frequently extends to the basal layer as the basal keratinocytes may be destroyed by infiltrating lymphocytes. Necrotic basal keratinocytes form colloid bodies or "Civatte bodies" with lymphocytic infiltrates forming satellites about them. In more advanced lesions, the lymphocytic infiltrates become so dense that the epithelial and connective tissue junction is obscured.

The histological diagnosis criteria established by Krutchkoff and Eisenberg (1985) include essential features, additional features, and disqualifying features. The essential features of OLP are:

(1) liquefactive degeneration or destruction of basal cells

(2) bandlike infiltrate of lymphocyte within lamina propria that intimately intermingles with the basal cell region of surface epithelium

Additional features which may or may not be present included:

(1) "saw-toothed" rete ridges

(2) hyperorthokeratosis or hyperparakeratosis

(3) separation of epithelium from the connective tissue with ragged cleavage and

(4) formation of Civatte bodies.

Disqualifying features are:

(1) presence of topographic and cytologic features of dysplasia

(2) presence of heterogeneous round cell infiltrate within the lamina propria

(3) diffuse extension of infiltrate into deeper submucosal tissues or frank preinvasive distribution of infiltrate.

3. GENETIC CHANGE AND TUMOURIGENESIS

3.1. Oncogenes and Suppressor Genes

In 1994 Vogelstein developed a model for the development of colon cancer which linked the histological changes that occur in that tissue during the development of cancer to mutation of specific genes. The underlying implication of this model was that for cancer to develop, a critical number of genetic changes had to occur in the tissue and that these changes accumulate during the progression of the tumour and are integral to the development of the pathology.

Since that time, progression models have been proposed for other types of cancers including head and neck tumours (Califano *et al*, 1996). A current estimate is that 7-10 independent genetic

events are involved in the production of invasive SCC in the head and neck region (Renan 1993). These genetic events include the mutation of a number of critical genes that control the processes of cellular proliferation and differentiation in a tissue. These genes associated with carcinogenesis are classified into two main groups: oncogenes and tumour suppressor genes.

Oncogenes are derived by the mutation of normal cellular genes termed protooncogenes. These genes are positive regulators of (act to stimulate) critical cellular processes such as cell proliferation. Many protooncogenes code for proteins that are involved in signal transduction pathways, eventually controlling the transcription of genes with products that are essential for triggering cell turnover. Approximately 50 different oncogenes have been identified, coding for proteins that function as growth factors, growth factor receptors, cytoplasmic second messengers and regulators of gene expression.

Very few oncogenes have been identified in head and neck cancers. Among the genes mutated are *ras*, *myc*, *erb*B-1 (epidermal growth factor receptor), *erb*B-2, *bcl-1*, and *int-2* (Kiaris *et al* 1995; Saranath *et al* 1993; Warnakulasuriya *et al* 1992; Lese *et al* 1995). Our knowledge of the frequency of mutation of these genes in different populations is still somewhat limited. For example, *ras* and *myc* mutation appear to be more prevalent in head and neck tumours occurring in the Far East, possibly due to the use of chewing tobacco and betel quid by these populations (Paterson *et al* 1996, Saranath *et al* 1993, 1994). 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. It is not known whether this effect was due

to a mutation of the gene itself or due to a dysregulation of the expression of this gene resulting from a downstream effect of another mutation.

In contrast to oncogenes, tumour suppressor genes (TSG) code for proteins that are negative regulators of (act to block) critical cellular processes such as cell proliferation. The functions of TSGs must be lost in order for tumourigenesis to occur. According to Knudson's hypothesis (1985), both copies of a tumour suppressor gene have to be inactivated for its protective functio to be lost in a cell. Experience with known suppressor genes, such as the retinoblastoma gene, suggests that this process involves two separate events, the first quite often involving a point mutation in one allele, followed by loss of loci containing the wild type gene in the remaining allele. Recently, powerful new procedures have been developed to identify the loss of these critical loci on specific chromosomes even from archival material. One technique is referred to as microsatellite analysis, which detects loss of heterozygosity (LOH) and is a useful tool for determining the loss of putative TSGs.

3.2. Microsatellite Loss of Heterozygosity Assay

Microsatellite analysis has been shown to detect allelic loss in very small amounts of DNA (ng quantities). This characteristic is essential when working with premalignant lesions, since these samples are rare and biopsies are small in size.

Microsatellites are tandem repeats of di, tri, or tetranucleotides, which are highly polymorphic an well-distributed throughout the human genome. They are useful markers for studying genetic change because they show high levels of heterozygosity (60-80%). This means that human

populations have evolved variants of these regions, differing in the number of repeats present within the microsatellite region. Informative cases will have a different number of nucleotide bases in the maternal and paternal copies of the region.

Microsatellite LOH analysis begins with the amplification of DNA by the polymerase chain reaction (PCR) employing ^{32}P end-labelled primers that flank an area of tandem repeats in the chromosomal region of interest. Two samples of DNA are amplified at the same time in separate reactions: DNA extracted from cells in the lesion of interest (e.g. epithelial cells in the tumour or premalignant lesion) and DNA extracted from normal tissues, the latter usually obtained from connective tissue underlying the lesion, or where possible, lymphocytes isolated from blood samples. After amplification, the PCR products are separated on a denaturing polyacrylamide gel and exposed to autoradiographic film. A typical informative sample will yield two alleles in the control tissue, one of maternal and another of paternal origin, with electrophoretic migration dependent on the allele size. Samples are scored by comparison of bands of lesion DNA with DNA of its corresponding normal control. LOH is reported if an allele is at least 50% lost in the lesion sample compared to the control. A sample is non-informative if both parental alleles are of the same size.

3.3. LOH in Head and Neck Carcinogenesis

3.3.1. Tumour and LOH

The majority of molecular studies on oral squamous cell carcinomas have not been restricted to the oral cavity but instead have included tumours falling into the broader category of head and neck squamous cell carcinomas (HNSCC). By definition, the oral cavity would include the inner mucosa of the lip, the buccal mucosa, tongue, palate, floor of mouth, and gums. The broader classification of HNSCC would also include the salivary glands, oropharynx, nasopharynx, and hypopharynx. The following summary describes studies performed with microsatellite markers i HNSCC, but where possible data will be presented specifically for the oral cavity (Table 2). Sinc our study group was centered on oral lesions, a better comparison would be with the latter topographical grouping.

In general, genetic studies of HNSCCs have shown that there are multiple regions of chromosomal loss in these tumours. These studies have been done in two main ways. A few studies have included a complete allelotyping of the tumour DNA using primers for each chromosomal arm (Ah-See *et al* 1994; Nawroz *et al* 1994; Field *et al* 1995). These studies have resulted in the identification of chromosomal regions that are most frequently lost in these tumours. Other studies have focused on these regions using more samples and in some cases increasing the number of primers to better define the region of loss.

Table 2 presents the data obtained on chromosomal regions most frequently lost in HNSCCs and oral tumours. For each study shown in the table, the data are presented as a ratio of the number of cases showing LOH at the indicated region compared to the total number of informative cases

In addition, the percentage of informative cases showing LOH has been calculated for each study and the range of these frequencies is presented in bold type for each chromosomal arm.

Although there is a large variation in frequencies among the studies, there is a general consensus that the most commonly observed regions of loss in these tumours occurs on 9p, 3p, and possibly 17p. These data suggest that these regions contain loci that are critically involved in tumourigenesis, probably early during the process. Other regions of LOH may be specifically associated with later events that are specific to the clinicopathological characteristics of the tumour. For example, El-Naggar *et al* (1995) suggest that LOH at 8p may be more frequently associated with high stage tumours.

Table 2. LOH Frequencies in Head and Neck or Oral Cancers

Chromosome Arm	Oral Cancer	Head and Neck Cancers
3p	14/27 Wu *et al* 1994 26/45 Ishwad *et al* 1996 **52-58%**	8/18 Ah-See *et al* 1994 32/61 Adamson *et al* 1994 9/19 El-Naggar *et al* 1995 18/27 Califano *et al* 1996 18/27 Nawroz *et al* 1994 22/46 Rowley *et al* 1996 **44 - 67%**
3q	N/A	4/32 Field *et al* 1995 **13%**
5q	N/A	12/28 Ah-See *et al* 1994 5/20 Nawroz *et al* 1994 13/45 Field *et al* 1995 4/19 El-Naggar *et al* 1995 **21 - 43%**
8p	N/A	14/40 Field *et al* 1995 10/19 El-Naggar *et al* 1995 8/20 Califano *et al* 1996 11/36 Li *et al* 1994 8/20 Nawroz *et al* 1994 **31 - 53%**
9p	35/73 Ishwad *et al* 1996 **48%**	24/39 Field *et al* 1995 13/18 El-Naggar *et al* 1995 54/74 Califano *et al* 1996 21/29 van der Riet *et al* 1994 21/29 Nawroz *et al* 1994 **62 - 73%**
9q	N/A	6/17 Ah-See *et al* 1994 6/30 Field *et al* 1995 7/20 El-Naggar *et al* 1995 3/23 Nawroz *et al* 1994 **13-35%**

Table 2. Continued

11p	N/A	5/39 Field *et al* 1995 4/24 Nawroz *et al* 1994 **13-17%**
11q	N/A	9/20 Ah-See *et al* 1994 3/39 Field *et al* 1995 5/15 El-Naggar *et al* 1995 14/23 Nawroz *et al* 1994 **23-61%**
13q	N/A	8/30 Field *et al* 1995 31/60 Califano *et al* 1996 12/22 Nawroz *et al* 1994 16/60 Li *et al* 1994 **27-54%**
17p	N/A	6/19 Ah-See *et al* 1994 19/38 Adamson *et al* 1994 18/36 Field *et al* 1995 3/14 El-Naggar *et al* 1995 34/62 Califano *et al* 1996 9/20 Li *et al* 1994 12/22 Nawroz *et al* 1994 **21-55%**
17q	N/A	12/40 Field *et al* 1995 4/18 El-Naggar *et al* 1995 8/26 Nawroz *et al* 1994 **22-31%**
18p	N/A	5/31 Field *et al* 1995 3/18 El-Naggar *et al* 1995 6/22 Nawroz *et al* 1994 **16-27%**
18q	N/A	20/41 Rowley *et al* 1995 4/19 El-Naggar *et al* 1995 6/26 Nawroz *et al* 1994 **21-49%**
19q	N/A	10/25 Nawroz *et al* 1994 **40%**

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

3.3.2. Dysplasia and LOH

Most tumours are genetically unstable and contain multiple genetic changes and much clonal heterogeneity. Therefore it is difficult to use these data to determine which genetic changes actually drive the process of carcinogenesis. In other words, tumour studies yield a limited amount of information on the temporal patterns or stages at which specific alterations occur during the multistage tumourigenesis. Since carcinogenesis involves the sequential accumulation of genetic alterations, studies on earlier lesions would better define those changes associated witl the development and progression of HNSCCs. Unfortunately, studies on earlier dysplastic lesion have been limited by the comparative difficulty of obtaining and working with such samples, which are usually much smaller than tumour samples.

Although the majority of studies have been performed on advanced, invasive tumours, a few studies have examined LOH frequencies in premalignant lesions. Table 3 summarizes the studies done on preinvasive lesions of the oral cavity specifically and on head and neck sites in general. These studies used a limited number of samples (all of which were microdissected) and focused mainly on those chromosome regions previously shown to sustain the greatest frequency of loss i HNSCCs.

Table 3. LOH Frequencies in Dysplastic Lesions

Chromosome arm	Oral Cancer	Head and Neck Cancers
3p	7/21 Roz *et al* 1996 6/32 Mao *et al* 1996 12/30 Emilion *et al* 1996 **19-40%**	15/29 Califano *et al* 1996 1/19 El-Naggar *et al* 1995 **5-52%**
8p	N/A	3/24 Califano *et al* 1996 **13%**
8q	N/A	6/28 Califano *et al* 1996 **21%**
9p	12/32 Mao *et al* 1996 **38%**	17/30 Califano *et al* 1996 6/18 El-Naggar *et al* 1995 **33-57%**
9q	N/A	2/20 El-Naggar *et al* 1995 **10%**
11q	N/A	9/31 Califano *et al* 1996 1/15 El-Naggar *et al* 1995 **7-29%**
13q	N/A	9/28 Califano *et al* 1996 **32%**
17p	3/16 Emilion *et al* 1996 **18%**	10/30 Califano *et al* 1996 1/14 El-Naggar *et al* 1995 **7-33%**
18q	111 Emilion *et al* 1996 **9%**	4/19 El-Naggar *et al* 1995 **21%**
19q	N/A	N/A

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

There are some major limitations in these studies. As shown in Table 4, most of these studies only examined the more advanced premalignant lesions, limiting the study cases to severe dysplasia and c.i.s. (El-Naggar *et al* 1995; Roz *et al* 1996). In the study reported by El-Naggar *et al* (1995) these dysplastic lesions were restricted to specimens obtained from patients that had a concurrent SCC. Genetic changes present in dysplastic lesions in the absence of SCC have been shown to

differ significantly from those lesions from patients with an invasive component, either concomitant or in a previous biopsy (Emilion *et al* 1996). The study by Mao *et al* reported on tl frequency of leukoplakias that had 3p or 9p loss but gave no indication of the degree of dysplasi: in the lesion. Two additional studies (Califano *et al* 1996; Emilion *et al* 1996) grouped all dysplastic lesions together with no indication of the severity of these lesions. A final study by Emilion *et al* (1996) did use dysplastic lesions with a range of severity but the number of lesions studied in the absence of SCC was small (8 mild, 4 moderate, and 5 severe) and the data are presented in a form that makes it difficult to interpret whether the severity of the lesion affected the reported LOH frequencies. However despite these limitations there is a general consensus in these studies that 3p and 9p represent the earliest and most frequent LOH changes in these early lesions.

Table 4. LOH and Degrees of Oral Dysplasias

Authors	LOH Frequency	Classification of Lesion
Califano *et al* 1996 (Head & Neck)	3p - 15/29 **(52%)** 9p - 17/30 **(57%)** 17p - 10/30 **(33%)**	Dysplasia with no indication of severity
El-Naggar *et al* 1995 (Head & Neck)	3p - 1/19 **(5%)** 9p - 6/18 **(33%)**	Severe/cis lesions only
Mao *et al* 1996 (Oral)	3p - 6/32 **(19%)** 9p - 12/32 **(37.5%)**	Reported as leukoplakias - some of which were dysplastic
Emilion *et al* 1996 (Oral)	3p - 7/17 **(41%)** 17p - 3/17 **(18%)** (only lesions without associated SCC included)	Used different levels of dysplasia but LOH results were not shown for the different degrees of dysplasias
Roz *et al* 1996 (Oral)	3p - 7/21 **(33%)**	Severe/*cis* lesions only

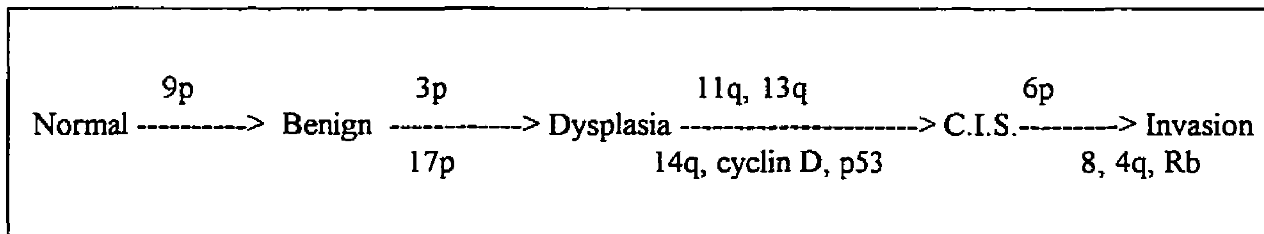
Data shown as number of lesions showing LOH/total number of informative cases. Numbers in bold face are the range of %LOH for indicated chromosomal arm.

In summary, information on LOH in different degrees of oral dysplasias (different stages of early oral carcinogenesis) is lacking. In addition, there is no study of genetic changes in OLP.

3.3.3. Tumour Progression Model for Head and Neck Cancers

Progression models are used to correlate genetic events with histopathological progression. Califano *et al* (1996) postulated a progression model for head and neck cancer based on LOH analysis of lesions ranging from benign squamous hyperplasia and dysplasia to c.i.s. (Figure 1).

Figure 1: Tumour Progression Model for Head and Neck cancers as proposed by Califano *et al* 1996.



According to this model, the loss of loci on 9p was the earliest event to occur and was present even in benign lesions. This loss was followed by LOH on 3p and 17p, then 11q, 13q, and 14q. As the lesion progressed from c.i.s. to invasive SCC, 5p, 8, and 4q were lost. Other studies on head and neck cancers are less extensive, using fewer premalignant lesions and/or limited to a smaller number of chromosomal arms. However, there is a general consensus that the earliest regions of loss in premalignant lesions occur on 3p and 9p followed by 17p (Califano *et al* 1996; Emilion *et al* 1996). These chromosomal arms also tend to have the highest frequencies of occurrence in head and neck and oral tumours. As this study centered on oral premalignant lesions (early carcinogenesis phases), it makes sense to focus at least initially on studying loss of

these 3 chromosomal arms in these lesions. The following section will briefly describe our presen state of knowledge on chromosomal loss on these 3 chromosomal arms in head and neck tumours

3.3.4. LOH Analysis at 3p, 9p, and 17p in Head and Neck Tumours

Chromosome 3p. Chromosome 3p loss is a common occurrence in many types of tumours including lung, kidneys, cervix, ovary, endometrium, and breast. (Wu *et al* 1993). It has been reported in approximately two-thirds of head and neck squamous cell carcinomas (Ishwad *et al* 1996; Nawroz *et al* 1994). Fine mapping of the area of loss in these tumours has led to data that support the involvement of 3 discrete regions of deletion 3p13-3p21.1, 3p21.3-3p23, and 3p25 (Wu *et al* 1993, Maestro *et al*, 1993). A new putative tumour suppressor gene called *FHIT* has recently been localized to the 3p14.2 site. LOH in this region has been detected in cancers such as lung (Sozzi, *et al* 1996) and colon (Ohta *et al* 1996). A number of microsatellite markers are presently available that span the *FHIT* region.

Chromosome 9p. Allelic loss on the short arm of chromosome 9 has been reported in a number o tumours including the lung (Merlo *et al* 1994), bladder (Ruppert *et al* 1993), and head and neck. This region is commonly lost by homozygous deletion in many tumour types (Cairns *et al* 1996; Kamb 1994). Loss of this arm has been reported for more than two-thirds of head and neck tumours (Nawroz *et al* 1994; Field 1995; El Naggar *et al* 1995; Califano *et al* 1996; Li 1994). Not only is 9p loss one of the most frequently occurring allelic losses in these tumours, but also among the earliest changes present (Califano *et al* 1996; El-Naggar *et al* 1995; Mao *et al* 1996). The putative tumour suppressor gene in this region is near the interferon (IFNA) locus and is as yet unidentified although there is some support for the locus being that of the *p16/CDKN2/MTS1*

gene. This gene encodes an inhibitor (*p16*) of cyclin-dependent kinase 4, a kinase that is essenti
for progression of the cell through the G1/S phase transition point.

Chromosome 17p. LOH of 17p has been reported to occur in 45-50% of head and neck cancers (Li *et al* 1994; Adamson *et al* 1994). Most likely the gene lost in this region is *TP53*, a gene which codes for a protein that is involved in the surveillance and response of cells to DNA damage. *p53* acts as a transcriptional activator of a series of genes that code for proteins in pathways that deal with genetic damage. These proteins are involved in DNA repair (e.g. GADD45), cell cycle inhibition (e.g. *p21*), and apoptosis (e.g. *bcl-2*) (White 1996). The possibility that the gene lost in this region is *p53* is supported by data using the microsatellite marker *TP53*, an informative polymorphism within the *p53* gene. However, there is some suggestion that a novel tumour suppressor gene besides *p53* may also be involved, and it is located in the region defined by the cholinergenic receptor B1 (CHRNB1) locus at 17p12-p11 (Adamson *et al* 1994).

3.4. Genes on Chromosomes 3p, 9p, and 17p

3.4.1. The *p53* Gene

Mutation of the *p53* gene accompanied by a loss of the other *p53* allele (located on chromosome 17p13) is the most common genetic alteration in human cancer (Hollstein *et al* 1991; Nigro *et al* 1989).

The *p53* protein was discovered in 1979 as a phosphoprotein which co-immunoprecipitated witl SV40 T-antigen (Lane and Crawford 1979). Due to the elevated levels found in dividing and transformed cells, *p53* was classified as an oncogene until its true nature as a tumour suppressor was discovered. Hinds *et al* (1989) reported that the *p53* protein previously detected was actuall a mutated form and that the wild-type form acted as a negative cell cycle regulator (Diller *et al* 1990; Baker *et al* 1990).

In response to DNA damage, the *p53* protein normally functions as a negative regulator of the c cycle by causing the cell cycle to arrest in the G_1 phase (to give the cell time to repair itself) or b inducing apoptosis if repair fails. In a sense it functions to preserve genomic integrity. For example, in response to ionizing radiation, *p53* is induced and activates the expression of the *p2* gene. The *p21* protein is thought to induce cell-cycle arrest through the inhibition of cyclin-dependent kinase complexes. These kinases allow the cell to pass from G_1 into S phase by phosphorylating critical substrates. The inhibition of kinase activity prevents the cell's entry into S phase. Wild type *p53* protein is difficult to detect immunohistochemically due to its low concentration and short half life. Consequentially, most forms of *p53* detected in this manner are mutated forms, which have a much longer half life. Mutation of *p53* may result in no expression of wild-type *p53*, or more commonly an abnormal protein that not only fails in its function of DNA repair, but also forms complexes with normal wild type *p53* hindering its function.

p53 mutations usually are missense mutations which result in a substituted amino acid and thus a altered protein. Other mutations such as nonsense and frameshift mutations occur occasionally and the proteins these mutant genes encode are undetectable by immunohistological methods due to their unstable nature.

Mutations in the *p53* gene and over-expression of *p53* protein have been reported in a large number of human cancers, including both carcinomas and sarcomas, and are thought to be the most common alteration in human cancer. There have been a number of studies investigating the *p53* protein over-expression in oral premalignant and malignant lesions. Several papers have shown that a high percentage of oral SCC stained positive for the mutated *p53* products. For example, Kaur *et al* (1994) detected positive *p53* immunostaining in 55% of oral dysplasias and in 75% of oral SCC. When the stage-wise progression of the oral lesions is considered, the studies have found that with increasing degrees of dysplasia and the appearance of SCC, not only was there an increase in the percentage of cases demonstrating *p53* staining, but also an increase in the staining intensity of the positive cells and expansion of these positive cells (Zhang *et al* 1993).

Several studies have measured the rate of *p53* mutation in oral cancer and HNSCC. Ahomadegbe *et al* (1995) detected mutation in 69% of lesions studied, with similar results from other studies (Boyle *et al* 1993; Zariwala *et al* 1994; Saranath *et al* 1993; Greenblatt *et al* 1994). A high percentage of HNSCC was found to contain *p53* mutation although the percentage of *p53* mutation in SCC was lower than that of *p53* protein over-expression. The significance of this discrepancy remains unknown and it is possible that mechanisms other than gene mutation may be responsible for the *p53* over-expression.

Few studies, however, have investigated *p53* mutations with respect to preinvasive oral lesions, particularly the early dysplastic lesions or in a stagewise fashion. In a study by Lazarus *et al* (1995), 4 of 26 cases of oral premalignant lesions demonstrated *p53* mutation. Of the four lesions

showing mutation, 1 was from mild dysplasia, 1 from moderate dysplasia, and 2 from severe dysplasia, suggesting that *p53* mutation may be a very early event in HNSCC carcinogenesis.

3.4.2. The *FHIT* Gene

Fragile sites are portions of chromosomes that are extremely weak and break easily. The nature of these sites has led to speculation that they might be involved in genetic instabilities that arise i tumours. These weak areas may also be an easy target for carcinogens such as those found in tobacco. In 1996 Ohta *et al* discovered a gene which they called *FHIT* (fragile histidine triad) located on 3p14.2, one of the most common fragile sites on 3p. This gene was missing wholly o partly in a wide array of tumours, namely, colon, breast, and lung (Ohta *et al* 1996; Sozzi *et al* 1996). Alterations such as homozygous deletions, aberrant transcripts, and LOH of the *FHIT* gene have been detected in HNSCC cell lines (Virgilio *et al* 1996, Mao *et al* 1996). The function of *FHIT* is yet to be elucidated but it has been found to be partly homologous to a yeast enzyme that functions in cell cycle control (Ohta *et al* 1996).

3.4.3. The *p16* Gene

The Multiple Tumour Suppressor 1 (*MTS1*) gene, otherwise known as the *p16* gene, is located o the short arm of chromosome 9. In order to understand the function of the *p16* protein, it is necessary to have some basic knowledge of the cell cycle. The cell cycle is the interval between two consecutive cell divisions and is divided into two major phases: interphase and mitotic periods. Interphase, which in turn is divided into G_1, S, and G_2 phases, comprises the majority of the cycle. The G_1 (gap 1) phase is simply the regular synthetic and biologic activities of the cell.

Next is the DNA synthesis phase, or S phase, where DNA replicates itself, and is followed by th
G_2 phase where there is a tetraploid number of cells which allows the mitotic (M) phase to occu
The transition from G_1 to S phase is the most important portion of the cell cycle in tumourigenesis.

Cyclin dependent kinase 4 (CDK4) along with cyclins and its other CDK counterparts are responsible for cell cycle regulation. In order for a cell to progress from G_1 to S phase, the activity of the complex cyclin D1/CDK4 is required. Known to be linked to the cell cycle, the *p1* protein has been identified as an inhibitor of cyclin-dependent kinase 4 and thus interrupts the cell's entry into S phase. Unlike *p21* protein which inhibits cyclin/CDK complexes, the *p16* protein inhibits CDK4 kinase activity directly (Serrano *et al* 1993). The *p16* gene has recently been mapped to the 9p21 locus, the same site as the *MTS1* (multiple tumour suppressor) gene. (Serrano *et al* 1993; Kamb *et al* 1994). Mutations or deletions of *p16* have also been discovered in various different tumour cell lines including breast, lymphoma, and bladder.

Tam *et al* (1994) studied the differential expression of *p16*. Comparing *p16* and *p21*, it was determined that *p16* was expressed at various levels in tumour cells while *p21* was ubiquitous. I some cell lines, *p16* was not detected at all, perhaps due to a deletion of its gene. When compared to the normal counterparts of the tumour lines, the levels of *p16* were much lower. Another interesting finding was that high levels of *p16* protein was positively correlated with inactivation of the retinoblastoma gene product. Kato *et al* (1993) suggested that high levels of the cyclin D/CDK4 gene product could also inactivate the retinoblastoma gene product. Tam *et al* measured *p16* protein expression during the cell cycle and found that the protein levels varied

during S phase, suggesting that *p16* functions in CDK4 inhibition only at the G1-S transition when CDK4 kinase is no longer needed.

Using glioma tumour cell lines, He *et al* (1994) determined that overexpressed CDK4 gene acts in a similar function to deletion of the *p16* gene. In tumour cell lines where *p16* genes were unaffected, high levels of CDK4 was reported. Increased CDK4 activity may negate the functio of *p16* protein resulting in tumour development.

3.5. The *p53*, *FHIT*, *p16* Genes, and Tobacco Usage

It is well known that carcinogens such as tobacco have a profound effect on the pathway or genetic nature of cancer development. Since oral cancer is mainly caused by the use of tobacco products, the following section will review the available literature on the relationship between th *p53*, *FHIT*, and *p16* genes and the use of tobacco products. There are not many examples in the literature relating genetic changes, particularly LOH, with tobacco use in HNSCC or dysplasias (Mao *et al* 1996; Partridge *et al* 1994; Lydiatt *et al* 1994). The apparent lack of information regarding the relationship between genetic changes and tobacco usage is due to several factors, namely, that most publications did not obtain or publish this information. In many studies, the smoking habit for the cases studied were largely unknown. In other studies, the subjects include were mainly smokers with an apparent lack of a sufficient number of non-smoker subjects for comparative purpose.

Measuring allelic loss on chromosome arms 3p, 5q, 9p, 9q,10q, 11p, 13q, 17p, and 18q of HNSCC samples, Lydiatt *et al* (1994) predicted the relationship between LOH, tobacco exposur

and early recurrence. Their surprising results showed that all non-smokers showed LOH on one or more loci compared to smokers who only demonstrated an LOH of 53%. Since multiple deletions have a higher rate of early recurrence, this outcome was predicted for the nonsmokers tested. According to Law (1990), only 15% of people who smoke develop lung cancer. Therefore, other factors besides exposure to tobacco must increase risk for HNSCC. Lydiatt suggested that possible explanations for these higher rates in nonsmokers could be unrecognized risk factors such as environment or a genetic predisposition to HNSCC.

Of the three genes to be reviewed here, the relationship between *p53* mutation and tobacco usage has been well studied while those of the other two genes are less known.

3.5.1. The *p53* Gene and Tobacco Usage

Mutation of the *p53* gene and overexpression of the *p53* protein are strongly correlated with the use of tobacco products. They also have been found in high frequency in those cancers that are strongly related to the use of tobacco products, such as head and neck SCC, a lower frequency in those cancers moderately related to the use of tobacco products, such as bladder cancer, and rare in those cancers that are not related to the use of tobacco products, such as sarcomas. Studies have shown that tobacco usage not only affects the incidence of *p53* mutation, but also has an effect on the type of *p53* mutations. The most common *p53* mutations are transversions or transitions of the base guanine (Raybaud-Diogene *et al* 1996; Ahomadegbe *et al* 1995; Boyle *et al* 1993).

For example, Lazarus *et al* (1995) measured *p53* mutations of oral premalignant lesions in non-smokers and smokers by PCR/SSCP analysis. All cases that demonstrated missense mutations in *p53* were from tobacco users, whereas non-tobacco users did not exhibit any *p53* mutations. In addition, ex-smokers also had a higher incidence of *p53* mutations than those who had never smoked.

The loss of function of a TSG is frequently associated with mutation of the TSG at one allele and LOH at the other allele. Since *p53* gene mutation is strongly associated with the use of tobacco products, one natural assumption is that LOH at 17p should also correlate with the use of tobacco products. However, information on the use of tobacco products and LOH at 17p is sparse. One study on lung cancer found that allelic loss at 17p was not correlated with smoking (Sato *et al* 1994). In another study on bladder cancer, neither *p53* mutation nor LOH at 17p was found to be significantly correlated with the smoking habit (Uchida *et al* 1995). Information on LOH at 17p in head and neck SCC is totally lacking.

3.5.2. The *FHIT* Gene and Tobacco Usage

The *FHIT* gene was only recently identified, and thus few studies are available regarding its characteristics, in particular its relationship to tobacco usage. Some presume that it may be associated with tobacco usage judging from the fact that genetic changes in this gene have been found frequently in HNSCC and other tobacco-induced malignancies (Schantz, 1994).

There are 4 studies available regarding the relationship of LOH at 3p where the *FHIT* gene is located and the use of tobacco products. The results are contradictory.

In a lung cancer study, allelic loss at 3p was not associated with the use of tobacco (Sato *et al* 1994). In two studies involving oral lesions, LOH at 3p was not found to be correlated with the use of tobacco products in either oral premalignant lesions (Mao *et al* 1996), or in oral SCC (Partridge *et al* 1994).

On the other hand, another study by Mitsudomi *et al* (1996) of non-small cell lung carcinomas (NSCLC) compared the LOH of 3p on smokers with that of non-smokers. The results showed a significant difference 36/68 (53%) loss of 3p in smokers as compared to 7/26 (27%) in non-smokers, (p=0.0235, chi squared test).

3.5.3. The *p16* Gene and Tobacco Usage

Again, there are few studies that have investigated the relationship of either *p16* gene, its protein products, or LOH at 9p where the *p16* gene is located with the use of tobacco products. In one study (Wu *et al* 1997) chromosome 9 aberrations were not found to be correlated with the use of tobacco products in lung carcinoma patients with a family history of cancer. In another study in which the *p16* protein expression status was investigated in lung carcinomas, the study found no correlation between loss of *p16* protein expression status and heavy smoking (Kinoshita *et al* 1996). In a study by Takeshima *et al* (1996), mutations of *p16* were investigated in lung adenocarcinomas of non-smoking Japanese women. No somatic mutations of *p16* were detected (except for two silent germline mutations in one case) in the 28 cases studied, again suggesting that *p16* gene change was not correlated with the use of tobacco products.

Two studies have investigated allelic loss at 9p and its relationship with the use of tobacco products. In a study using lung carcinomas, Sato *et al* (1994) compared LOH frequencies of smokers with that of non-smokers in adenocarcinoma of the lung. The results showed LOH at 9 was higher in smokers than in non-smokers but this difference was not statistically significant. I a study by Mao *et al* (1996) using oral premalignant lesions, again LOH at 9p was not found to b correlated with smoking.

4. STATEMENT OF PROBLEMS

4.1. Lack of Genetic Markers that Can Be Used to Predict Cancer Risk

The majority of oral premalignant lesions do not progress into cancer. The malignant potential o oral premalignant lesions are currently judged solely by the degree of dysplasia. According to th severity of dysplasia present in a lesion, oral premalignant lesions can be classified histologically into mild dysplasia, moderate dysplasia, severe dysplasia, and finally c.i.s. There are two main problems, however, with the use of dysplasia in predicting malignant potential of a lesions. First, while histological diagnosis and degree of dysplasia have a good predictive value for late preinvasive oral lesions (severe dysplasia and c.i.s.) since most of these lesions will progress into cancer, their predictive value for the early premalignant lesions (mild dysplasia and moderate dysplasia) is poor since most of these lesions do not progress into cancer. It is conceivable that these phenotypically similarly dysplastic lesions are genetically different, resulting in progression of some lesions while others do not progress. It is desirable that genetic markers be developed sc that those high risk lesions can be identified. Another problem with the histological method is tha it is unreliable due to its subjective nature. Discrepancies in diagnoses are frequently found

between pathologists and certainly between different medical centres. Again, the development of more objective methods, such as genetic markers, in risk prediction for oral premalignant lesions is highly desirable.

Recently, new procedures have been developed to identify the loss of critical loci on specific chromosomes even from archival material. These loci contain putative TSGs that must be lost for tumourigenesis to occur. One technique, microsatellite analysis, detects loss of heterozygosity (LOH), and is a useful tool for determining the loss of putative TSGs. LOH has been found to be a common event in many head and neck cancers, particularly on chromosomes 3p, 9p, and 17p. However, information on LOH in oral premalignant lesions (oral dysplasias) is sparse, and there is a lack of information regarding loss of tumour suppressor genes and progression of oral premalignant lesions. This study investigated allelic loss on chromosome arms 3p, 9p, and 17p using different stages (degrees) of oral dysplastic lesions and SCC. It was hoped that the study would shed light on the temporal progressive development of genetic changes during oral carcinogenesis, and that these genetic changes may be used in cancer prediction of oral precancerous lesions. In addition, the study should also add to our knowledge on the molecular mechanism of tumour progression and oral carcinogenesis.

4.2. Is Oral Lichen Planus a Premalignant Lesion?

OLP is one of the most common oral mucosa diseases, and is hotly debated regarding its malignant potential (Krutchkoff and Eisenberg 1985; 1992; Duffey *et al* 1996). Evidence supporting OLP as a premalignant lesion comes from epidemiological studies in which about 1-

2% of OLP were found to progress into oral SCC (Sigurgeirsson *et al* 1991; Murti *et al* 1986; Salem 1989; Holmstrup *et al* 1988). On the other hand, it was argued that OLP *per se* is not premalignant and only those OLP-like lesions that have dysplastic changes are premalignant (Krutchkoff and Eisenberg 1985; 1992).

Is it possible that only those OLP with dysplasia is precancerous? Is OLP (without dysplasia) precancerous? If cancer only develops from those cases of OLP-like lesions that demonstrate dysplasia, it seems unfair to let a large population of people with this common oral disease without dysplasia carry the long-term mental burden that they are suffering from a premalignant oral lesion. On the other hand, if OLP is indeed premalignant even without dysplasia, some OLP patients may not have been appropriately managed and followed up. Studies are highly desired o this issue.

One way to answer the above questions is to study the genetic changes in OLP and to determine those changes that are presumed to be of specific importance to early oral carcinogenesis (oral dysplasia) are found in OLP. There is a lack of study on genetic changes of OLP.

5. OBJECTIVES OF THE STUDY

(1) To obtain information on allelic loss on chromosomal arms 3p, 9p, and 17p in different degrees (stages) of oral dysplasias (premalignant lesions).

(2) To obtain information on allelic loss on chromosomal arms 3p, 9p, and 17p and to use th data to determine whether OLP show similar patterns of LOH to that of oral dysplasia.

6. HYPOTHESES TESTED

(1) To test the hypothesis that LOH on chromosome arms 3p, 9p, and 17p occurs in both dysplastic and cancerous oral lesions, and the frequency of such LOH increases with progression of the oral dysplastic lesions and the development of SCC.

If the data support the specific hypothesis, it would suggest that LOH at these chromosome arms plays an important part in early oral carcinogenesis, and the data may be used to establish the molecular progression model for oral carcinogenesis.

If the data disagree with the specific hypothesis, it would suggest that LOH at these chromosomes occurs late in oral carcinogenesis.

(2) To test the hypothesis that LOH on chromosome arms 3p, 9p, and 17p does not occur or occur rarely in OLP.

If the data support the specific hypothesis, it would suggest that OLP is not premalignant and that oral cancers reportedly arising from OLP were probably from lichenoid dysplasia instead. If the data disagree with the specific hypothesis and OLP is found to have similar frequencies of allelic loss as oral dysplasia, though to a lesser degree, it would suggest tha OLP is indeed an oral premalignant lesion, with or without dysplastic changes.

7. SIGNIFICANCE OF THE STUDY

The results provide information on the molecular genetic profiles of these oral premalignant lesions. Such information is currently limited but critical to our understanding of the mechanism of early carcinogenesis. In addition, the information generated may provide a better understanding of the malignant potential of early dysplastic lesions and OLP and hence assist the clinician in determining that appropriate treatment and management of these lesions is rendered.

8. MATERIALS AND METHODS

8.1. Samples

8.1.1. Sample Source

Archival paraffin blocks were obtained from the oral pathology division of the Vancouver Genera Hospital & Health Sciences Centre. The use of these samples was approved by the University Ethics Committee.

A total of 151 specimens were used for this study, and consisted of the following groups:

Group 1:	Reactive Lesions	29 cases
Group 2:	OLP	33 cases
Group 3:	Oral dysplasia	61 cases total
	Mild dysplasia	21 cases
	Moderate dysplasia	21 cases
	Severe dysplasia/c.i.s.	19 cases
Group 4:	SCC	28 cases

All reactive lesions, OLP, dysplasias, and c.i.s. specimens were primary lesions and taken from patients who had no prior history of head and neck cancer. Group 1 consisted of epulides, denture-induced or other trauma-induced fibroepithelial polyps, gingival hyperplasia, periodontitis, and mucocele. These reactive hyperplastic lesions demonstrate epithelial hyperplasia and/or inflammation and served as a negative control group to rule out the possibility that the genetic changes observed in other groups are attributed to increased cell proliferation or chronic inflammation instead of premalignancy.

8.2. Diagnostic Criteria for the Samples

The histological diagnosis criteria used for OLP and lichenoid dysplasia were those described by Krutchkoff & Eisenberg (1985) and for dysplasia by WHO (1978) and are summarized below. The criteria for OLP were covered previously in section 2.4.

Histological Diagnosis of Lichenoid Lesions

Lichenoid lesions used in this study are characterized by superficial band-like infiltrate in lamina propria composed chiefly of lymphocytes (lichenoid), with no epithelial dysplasia, but otherwise do not fit into the strict criteria of OLP.

Histological diagnosis of lichenoid dysplasia (Krutchkoff & Eisenberg, 1985)

Lichenoid lesion or OLP + any 2 or more of the following features of dysplasia:

- Increased nuclear size, usually manifest by increased N/C ratios
- Nuclear pleomorphism
- Nuclear hyperchromasis
- Disturbed or disorderly epithelial maturation
- Lack of cellular cohesion, often manifest by marked intercellular edema
- Increased or abnormal mitoses
- Blunted, club-shaped, or "tear drop" shaped rete pegs

Histological Diagnosis of Oral Dysplasia (WHO, 1978)

- Loss of basal cell polarity
- More than 1 layer of basaloid cells
- Increased N/C ratio
- Drop-shaped rete ridges
- Irregular stratification

- Increased and abnormal mitoses
- Mitotic figures in the superficial half of the epithelium
- Cellular pleomorphism
- Nuclear hyperchromatism
- Enlarged nucleoli
- Reduction of cellular cohesion
- Keratinization of single cells or cell groups in the spinous cell layer

Not all of the above changes are necessarily seen in each case of dysplasia. While a division into mild, moderate and severe dysplasia, and c.i.s. can be somewhat arbitrary, the histological diagnoses (including grading dysplasia) of the lesions were done independently by 2 oral pathologists, Dr. R. Priddy and Dr. L. Zhang.

The criteria mentioned above for OLP were strictly followed to ensure that cases selected as OL] demonstrated no dysplasia. Only those cases in which an agreement was reached on diagnoses between the two pathologists were used.

8.3. Clinical Information

The following clinical data were obtained for the cases studied: age and gender of the patients, anatomical location of the lesions, and tobacco usage. However, not all of the patients' charts indicated tobacco usage habits. The complete list of the clinical information for each case is included in the appendix as Table 8.

Since the objective of the study was to correlate the LOH with the diagnoses of the oral lesions, the selection of cases was primarily based on the diagnoses of the lesions. However, OLP cases were purposely chosen from a largely non-smoking population. The rationale was that in order t determine the malignant potential of OLP, extraneous factors, such as the effect of tobacco,

should be minimized. This ensured that any LOH was due to OLP exclusively and not to the effects of carcinogens found in tobacco.

The majority of dysplastic lesions and SCC were obtained from patients who used tobacco products. Of the 33 OLP specimens, 7 were from smokers, 25 from those with no history of tobacco usage, and 1 from a patient whose habits were unknown. Of the 29 cases of reactive lesions, 7 specimens were from smokers, 21 from those who did not use tobacco products, while the smoking habit of 1 subject was unknown. Smoking habits of patients are summarized in Table 5.

Table 5. Summary of Histological Diagnosis vs. Smoking Habits

Diagnosis/# cases	Smoker or Former Smoker	Never Smoked	Habit Unknown
OLP (33)	7	25	1
Hyperplasia (29)	7	21	1
Mild Dysplasia (21)	13	2	6
Moderate Dysplasia (21)	11	1	9
Severe Dysplasia/c.i.s. (19)	9	2	8
Squamous Cell Carcinoma (28)	13	6	9

8.4. Slide Preparation

8.4.1. Tissue Block Selection

When a case was chosen based on the histological diagnosis (i.e. when our two pathologists had agreed on the diagnosis), the tissue block for the case was removed from the archive, one 5 micron thick slide was cut and stained with hematoxylin and eosin (H&E staining described below), and reviewed for the following features:

a) The size of the specimens left in the tissue block.

As this study required multiple thick sections of tissue in order to obtain a sufficient amount of DNA, a reasonably large tissue specimen was required. Since oral biopsies tend to be small, a number of cases were disqualified for the study on the basis of insufficient size. The study was limited to those cases with sufficient tissue left in the blocks.

b) The amount of oral mucosa lining squamous epithelium and the underlying connective tissue.

This study required the presence of a reasonable amount of tissues for both the squamous epithelium (target of the study), and the non-epithelial mesenchymal tissue (control). Those case: showing an apparent lack of either tissue were disqualified, such as those cases with ulceration in which epithelial tissue was mostly destroyed, or those cases of shallow biopsies in which there was little connective tissue.

8.4.2. Cutting and Staining of Slides

For each tissue block, one slide with a 5 micron-thick section was submitted for H&E staining an then cover-slipped for reference. Serial thick sections (12 microns thick) were cut (about 15 slides per block) and also stained with H&E, but left uncoverslipped for later microdissection. The H&E staining procedure is as follows:

Hematoxylin and Eosin (H&E Staining)

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. Samples were deparaffinized by two changes of xylene for 15 minutes each followed by dehydration in graded alcohols (100%, 95, 70% ethanol), and hydrated by rinsing in

tap water. Slides were then placed in Gill's Hematoxylin for 5 minutes followed by rinsing in tap water and were then blued with 1.5% (w/v) sodium bicarbonate. After rinsing in water, slides were lightly counterstained with eosin, dehydrated, and cleared for coverslipping. Thick sections to be dissected were stained by the above procedure without the dehydration step, and air dried.

8.5. Microdissection and DNA Extraction

A vital component of this study is the use of microdissection to isolate specific cells in tissue sections for molecular analysis. This ensures that squamous epithelial cells are separated from other cells and also ensures that squamous epithelial cells taken for a given diagnosis indeed represent its diagnosis. It is well known that the severity of dysplastic changes frequently varies from region to region within a single section and the diagnosis of a tissue specimen is based on th most severe part of the lesion. Since determination of dysplasia and degree of dysplasia requires the expertise of a pathologist, the microdissection of the specimens were performed or supervisec by Dr. L. Zhang. Areas of dysplasia, OLP, or cancer were identified using H&E stained sections cut from formalin-fixed paraffin-embedded tissues. Epithelial cells in these areas were meticulously microdissected from adjacent non-squamous epithelium tissue or cells under an inverted microscope using a 23G needle. Genomic DNA from normal tissue was obtained by dissecting out the underlying stroma in these sections and used as control DNA for the case. A minimum of 50 sectioned nuclei were used for each PCR reaction to prevent false LOH signals resulting from truncation of the nuclei during sectioning.

8.6. Sample Digestion and DNA Extraction

The microdissected tissue was placed in an eppendorf tube and digested in 300 uL of 50 mM Tris HCl (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) proteinase K (0.5mg/mL) at 48°C for 72 or more hours. During incubation, samples were spiked with 20 uL of fresh concentrated proteinase K (20mg/mL) twice daily. The DNA was then extracted three times with PC-9, a phenol-chloroform mixture, and precipitated with 100% ethanol in the presence of glycogen. The

samples were then resuspended in LOTE, a low ionic strength Tris buffer, and submitted for quantitation.

8.7. DNA Quantitation

After DNA extraction, quantitation of DNA was performed using fluorescence analysis with the Picogreen kit (Molecular Probes). The method required two standard curves to be prepared using dilutions of a standard DNA solution mixed with the Picogreen working solution. The low standard curve ranged from 1 to 20 ng, while the high standard curve ran from 10 ng to 400 ng. Absorbance was read from a SLM 4800C spectrofluorometer (SLM Instruments Inc. Urbana, IL). Sample DNA concentration was then read from one of the standard curves depending on its absorbance. The sample DNA was then adjusted to 5ng/uL with LOTE buffer.

8.8. LOH Analysis

Following microdissection and DNA extraction, all samples were coded so that the analysis of LOH would be performed without knowledge of the sample diagnosis. DNA was analyzed for LOH on 3p, 9p, and 17p arms using microsatellite markers obtained from Research Genetics (Huntsville, AL) that mapped to the following regions: 3p14.2 (*D3S1234, D3S1300*), 3p25.1-25.3 (*D3S1110*), 9p21, (*INFA, D9S171, D9S1751, D9S1748*), 17p13.1 (*TP53*), and 17p11.1-12 (*CHRNB1*). (Our initial studies used two primers for each chromosomal arm: *D3S1234* (flanks *FHIT* gene) and *D3S1300* (*FHIT* gene) for arm 3p; *D9S171* and *IFNA* for 9p, and *TP53* and *CHRNB1* for 17p). Additional primers were used for those samples showing non-informativity for the above primers, increasing the number of cases with informativity and better localizing the region of loss.

The choice of these markers was based on allelotyping studies of HNSCC (Califano *et al* 1996; Ah-See *et al* 1994) and on preliminary studies on premalignant lesions. These markers are localized in regions previously shown to be frequently lost in head and neck tumours. The 9p21 locus has been linked to putative tumour suppressor *p16*, a gene that codes for a cyclin dependent

kinase inhibitor involved in regulation of the cell cycle. Three regions of loss have been identifie for 3p (3p13-14.2, 3p21.3, and 3p25). The 3p14.2 locus in particular demonstrates elevated LO in head and neck SCC, as well as being associated with risk of premalignant lesions progressing t SCC. The markers chosen focus on this particular area with another marker in the 3p25 telomeri region used as a comparison. On chromosome 17, two markers were employed. One is located within the *p53* gene (*TP53*), a tumour suppressor gene that is frequently lost or altered in variou cancers, and the other is located at 17p11.1-p12 (*CHRNB1*). Previous studies have shown that *CHRNB1* is sometimes lost in the absence of an LOH at *TP53*. The putative tumour suppressor gene in this region is *p53*, but there is some indication that a second tumour suppressor gene could be present in this region.

Prior to amplification, T4 polynucleotide kinase (New England BioLabs) was used to end label 100 ng of one primer from each microsatellite pair with [γ-^{32}P]-ATP (20 μCi, Amersham). PCR amplification was carried out in 5 μl reaction volumes 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 (GIBCO, BRL); and PCR buffer [16.6 mM ammonium sulfate 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM ß-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide]. PCR amplification was performed for 40 cycles: denaturation at 95°C for 30 seconds, annealing at 50-60°C for 60 seconds, and extension at 70°C for 60 seconds with a final extension at 70°C for 5 minutes. The PCR products were then diluted 1:2 in loading buffer and separated on an 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography. The films were then coded and allelic loss was scored without knowledge of sample diagnosis.

8.9. Scoring of LOH and Statistical Analysis

For informative cases, allelic loss was scored when the signal intensity was decreased by at least 50% as compared to its normal control counterpart from connective tissue DNA. All samples showing LOH were repeated in an independent amplification and rescored.

Fisher's Exact and Chi-square tests were used to compare the prevalence of chromosome loss between subgroups. Specifically, for a given pattern of chromosome loss, a binary outcome was used to indicate whether a specimen had this pattern. These statistical tests were used to compar the proportions of specimens having the specific pattern of chromosome loss. The trends of LOI with the progression of lesions were examined using Spearman rank correlation.

9. RESULTS

9.1. LOH at 3p, 9p, and 17p in Dysplasia and Cancer

Table 6 presents frequencies of allelic loss on 3p, 9p, and 17p for 33 cases of OLPs, 29 reactive oral lesions, 61 preinvasive lesions (including 21 mild epithelial dysplasias, 21 moderate dysplasias, and 19 severe dysplasias/c.i.s.), and 28 carcinomas. The data are presented as total allelic loss for each arm as well as the percentage of cases showing any allelic loss (3p, 9p, or 17p), or loss of more than 1 of these arms.

Table 6. LOH Results

Diagnosis	# of cases	Total allelic loss/informative case for indicated region (%)				
		3p	9p	17p	LOH at any arm*	LOH at >1 arm*
OLP	33	2/33 **(6)**	0/31 **(0)**	0/33 **(0)**	2/33 **(6)***	0/33 **(0)**
Reactive Lesions	29	3/26 **(12)**	1/26 **(4)**	0/29 **(0)**	4/29 **(14)***	0/29 **(0)**
Mild Dysplasia	21	6/20 **(30)**	7/20 **(35)**	0/20 **(0)**	9/21 **(43)***	4/21 **(19)**
Moderate Dysplasia	21	6/15 **(40)**	9/17 **(53)**	7/21 **(33)**	12/21 **(57)**	8/21 **(38)**
Severe Dysplasia/ c.i.s.	19	7/19 **(37)**	12/18 **(67)**	7/18 **(39)**	15/19 **(79)**	8/19 **(42)**
SCC	28	25/28 **(89)**	23/28 **(82)**	20/28 **(71)**	28/28 **(100)**	23/28 **(82)**

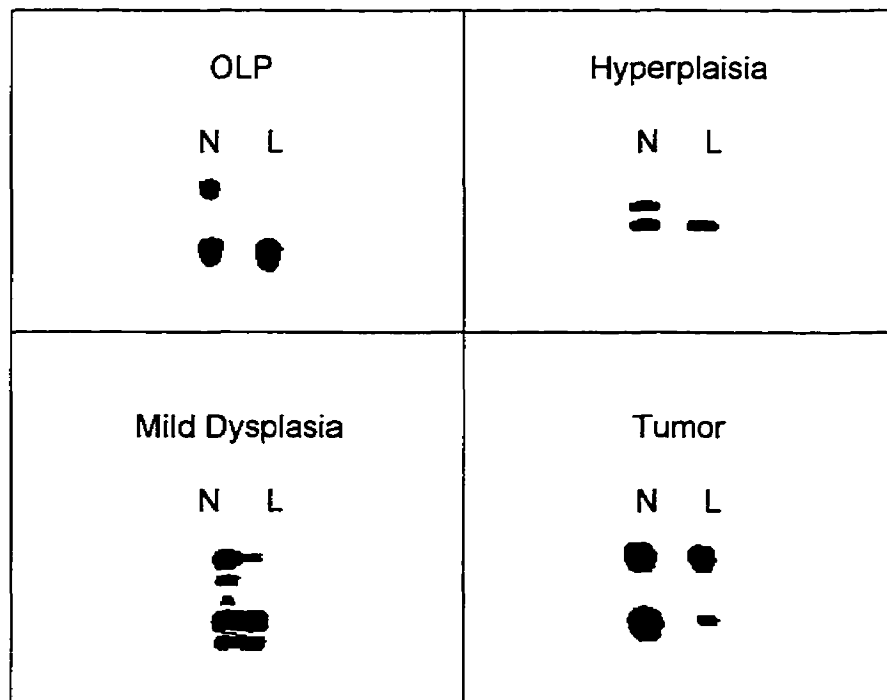
Frequency of loss correlated significantly with increasing degrees of dysplasia and progression into SCC for those lesions showing LOH at any arm ($p=0.0167$) and LOH at >1 arm ($p=0.0167$). Early mild dysplastic lesions showed significantly higher number of LOH on any arm than OLP ($p=0.0017$) and reactive lesions ($p=0.027$).

When the total allelic loss is considered, a low frequency of LOH was noted in the 29 non-premalignant reactive lesions (4/29, 14%), and LOH in all of the 4 cases were restricted to a single chromosome arm. This loss occurred at 3p14.1 for 3 cases and at 9p21 for 1 case.

In contrast, a much higher percentage of LOH were noted in oral dysplasias and carcinomas. LOH at either 3 chromosome arms was noted in 43% of mild dysplasias, 57% of moderate dysplasias, 79% of severe dysplasia/c.i.s. and 100% of SCC. The frequency of this loss correlate

significantly with increasing degrees of dysplasia and progression into carcinoma (p = 0.0167, Spearman Rank Correlation). Furthermore, LOH at multiple chromosome arms was also frequently noted in oral dysplasia and carcinoma. Multiple LOHs were found in 19% of mild dysplasia, 38% of moderate dysplasia, 42% of severe dysplasia/c.i.s., and 82% of carcinoma. T frequency of this multiple loss (multiple hit) again correlated significantly with increasing degree of dysplasia and progression into carcinoma (p = 0.0167, Spearman Rank Correlation). Representative LOH in OLP, hyperplasia, mild dysplasia, and tumour lesions are shown in Figure 2.

Figure 2: Representative LOH shown for various tissue types*



*N = DNA from normal tissue, L = DNA from lesion tissue

When the chromosome arms are considered individually, LOH at both 3p and 9p occurred in a significant percentage of oral mild dysplasia (30% and 35% respectively) whereas LOH at 17p d not appear until moderate dysplasia (Table 6). The frequency of LOH at 3p was similar among premalignant lesions, i.e., mild dysplasia (30%), moderate dysplasia (40%), and severe dysplasia/c.i.s. (37%) groups. There was a sharp rise of LOH at 3p with development of SCC (89%). For 9p, there seemed to be a steady increase of frequency of LOH with progression of th lesions: 35% of mild dysplasia, 53% of moderate dysplasia, 67% of severe dysplasia/c.i.s. and

82% of carcinomas. For 17p, LOH was not noted in mild dysplasia, but from moderate dysplasia there was a steady increase in the frequency of LOH with progression of the lesions: LOH was noted in 33% of moderate dysplasia, 39% of severe dysplasia/c.i.s., and 71% of carcinoma.

9.2. LOH at 3p, 9p, and17p in OLPs and Lichenoid Dysplasia

As shown in Table 6, only 6% of OLP showed LOH, a frequency that is even lower than that of the non-premalignant reactive lesions. Similar to reactive lesions, the allelic loss noted in OLP is again only present in a single chromosome arm. Two cases, 38LP and 40LP, (2/33, 6%) showed LOH. In both cases, the loss was confined to loci at 3p14.1. One case (40LP) displayed LOH at *D3S1234* and *D3S1300* while the other (38LP) showed loss at *D3S1300* but was non-informative at *D3S1234*. The pattern of loss was reproducible in 3 independent PCR reactions.

Clinically, both cases presented with classical features of OLP. Case 40LP, a 26 year old female, presented with diffuse white lacy lesions of long duration on the right buccal mucosa and the righ floor of the mouth which occasionally became erosive. Case 38LP, a 42 year old female, had white lesions on both right and left buccal mucosal surfaces. The biopsy sites for both of these cases was the right buccal mucosa.

One additional case involving a 47 year old man with a history of smoking was of interest. He presented clinically with multiple white oral lesions suggestive of OLP. Two concurrent biopsies from different oral sites were obtained. One biopsy showed classic OLP histologically while the other showed mild lichenoid dysplasia. For this case, further LOH analysis was performed on other loci commonly lost in head and neck tumours, namely, 4q, 8p, 11p, 11q, 13q, 14q, and 18q Although no LOH was found in the OLP biopsy, multiple regions of loss were present in the lichenoid dysplasia biopsy (9p, 4q, 13q, and 14q).

9.3. LOH in Relation to Smoking

9.3.1. LOH in OLP and Reactive Lesions in Relation to Smoking

Of the four non-premalignant reactive lesions that demonstrated LOH, two were from smokers and other two specimens were from patients who had never used tobacco products. Of the two cases of OLPs that demonstrated LOH, neither patient had a history of tobacco usage. The negative tobacco usage history was confirmed by telephoning the patients. The presence of LOH in these 6 cases in reactive lesions and OLPs was not associated with use of tobacco products: LOH was found in 10% (4/41 cases) of the non-tobacco users in these two groups, and also in 10% (2/21 cases) of the smokers.

9.3.2. LOH in Dysplasias and SCC in Relation to Smoking

As shown in Table 7, for 3p and 17p, smokers showed higher frequencies of LOH (52% and 66% respectively) than the non-smokers (37.5% and 43% respectively). However, the differences are not statistically significant. For 9p, both smokers and non-smokers showed approximately similar frequency of LOH (57% and 60% respectively).

Table 7. LOH in Oral Dysplasias and SCC of Smokers vs. Non-smokers

	Total allelic loss/informative cases for indicated region (%)				
Habit	3p	9p	17p	LOH any arm	LOH >1 arm
Smokers	23/44 **(52)**	24/42 **(57)**	21/32 **(66)**	30/44 **(68)**	23/44 **(52)**
Non-smokers	6/16 **(37.5)**	6/10 **(60)**	3/7 **(43)**	9/11 **(82)**	3/11 **(27)**

When LOH at any chromosome arm was considered, non-smokers showed higher frequency of LOH (82%) than the smokers (68%), but the difference is not statistically significant. On the other hand, when one looked at LOH at more than one arm, smokers showed higher frequency of LOH (52%) than the non-smokers (27%). Again the difference is not significant statistically.

10. DISCUSSION

10.1. LOH in Different Degrees of Oral Dysplasia

Premalignant lesions are those lesions that have an increased chance of cancer development as compared to their normal counterparts. The malignant potential of oral premalignant lesions are currently judged solely on the degree of dysplasias by examination of tissue sections stained with hematoxylin and eosin. According to the severity of dysplasia present in a lesion, oral premalignant lesions can be classified histologically into mild dysplasia, moderate dysplasia, seve dysplasia, and finally c.i.s. There are two main problems, however, with the use of dysplasia in predicting the malignant potential of a lesion. First, histological diagnosis and degree of dysplasi have a good predictive value for late preinvasive oral lesions (severe dysplasia and c.i.s.) since most of these lesions will progress into cancer. Their predictive value for the early premalignant lesions (mild dysplasia and moderate dysplasia) is poor since most of these lesions do not progre into cancer. It is conceivable that these phenotypically similarly dysplastic lesions are genetically different, resulting in progression of some lesions while others do not progress. It is desired that genetic markers be developed so that those high risk lesions can be identified. The second problem with the histological method is that this method is unreliable due to its subjective nature Discrepancies in diagnoses are frequently found between pathologists and certainly between different medical centres. Again, the development of more objective methods in risk prediction for oral premalignant lesions is highly desirable.

Recent studies of tumour progression models have shown that in some tumour types there is a correlation between specific genetic changes and histopathological progression. The identificatio of allelic losses in tumours within the context of a tumour progression model has led to the identification of critical putative tumour suppressor genes. Using a description of a molecular progression model of colorectal cancer Fearon and Vogelstein *et al* (1990) first indicated that

tumours progress via activation of oncogenes and the inactivation of TSGs. These genetic event: produce a growth advantage for a clonal population of cells. They also postulated that specific genetic events may occur in a distinct order of progression during multistep carcinogenesis.

Tumour suppressor genes play important roles in carcinogenesis and their functions must be lost in order for tumourigenesis to occur. Recent studies have shown that the loss of specific region: of chromosomes that contain tumour suppressor genes is a common event in malignant lesions. However, few studies have investigated these changes in premalignant lesions. Advanced, invasive neoplasms have multiple genetic changes and clonal heterogeneity which render the interpretation of results difficult. Without studying the premalignant lesions, the characteristic temporal pattern and/or stage at which alterations occur during the multistep carcinogenesis cannot be defined. Since tumourigenesis is a sequential accumulation of genetic alterations, analysis of the sequential stages of premalignant lesions is very important in identifying specific genetic changes that are associated with the development and progression of HNSCC. The identification of these genetic changes in premalignant lesions may be important in cancer risk prediction, management of the lesion and in understanding the mechanism of carcinogenesis.

LOH on chromosome arms 3p, 9p, and 17p in oral/head and neck SCC are well documented. 3p LOH on HNSCC or oral SCC ranges from 44-67% in the literature (Ah-See *et al* 1994; Adamso *et al* 1994; El-Naggar *et al* 1995; Califano *et al* 1996; Nawroz *et al* 1994; Wu *et al* 1994; Ishwa *et al* 1996). Loss on 9p is even higher, ranging from 48-73% (Field *et al* 1995; El-Naggar *et al* 1995; Califano *et al* 1996; van der Riet *et al* 1994; Nawroz *et al* 1994; Ishwad *et al* 1996). Few studies have investigated dysplastic lesions, and in these studies, the preinvasive lesions studied were usually exclusively late dysplastic lesions (severe dysplasia and c.i.s.) In one study, early dysplastic lesions were included; however, the results on LOH were not reported for these early lesions separately. Rather, the study grouped all the dysplastic lesions (Emilion *et al* 1996). Information on LOH at different stages of oral dysplasias was lacking.

In this study, we have investigated LOH at 3p, 9p, and 17p in oral premalignant lesions exhibitin different degrees of dysplasias and SCC. The study has, for the first time, shown that with progression of oral dysplasias comes a progressive development of LOH. The results have demonstrated that the frequency of cases with LOH involving a single chromosome arm increase with increasing degrees of dysplasia and progression into SCC (Table 6). LOH on either of the three arms was only present in 14% of reactive lesions, but increased with progression of the ora lesions and present in 43% of mild dysplasias, 57% of moderate dysplasias, 79% of severe dysplasia/c.i.s., and 100% of SCC. The frequency of this loss correlated significantly with progression of oral lesions ($p = 0.0167$). Even the early dysplastic lesion, mild dysplasia, showe significantly higher number of LOH cases than those of OLP ($p=0.0017$) and reactive lesions ($p=0.027$).

Furthermore, LOH was detected on only a single arm in reactive lesions but occurred on more than one chromosome (multiple hits) in dysplasia and cancer. Multiple chromosome loss was found in none of the reactive lesions but present in 19% of mild dysplasias, 38% of moderate dysplasias, 42% of severe dysplasia/c.i.s., and 82% of SCC. The frequency of this multiple loss (multiple hit) again correlated significantly with progression of oral lesions ($p = 0.0167$).

When the chromosome arms are considered individually, LOH at both 3p and 9p occurred in a significant percentage of oral mild dysplasia whereas LOH at 17p did not appear until moderate dysplasia (Table 6).

The above findings are important in our understanding of the progression of oral cancer. The results confirmed our hypothesis that with progression of oral premalignant lesions and transformation of premalignant lesions into carcinomas, there is a progressive loss of tumour suppressor genes. The results suggest that the earliest molecular events in oral cancer development include LOH at 3p and 9p, followed by LOH at 17p.

The findings also have important clinical implications. With rapid development of molecular biology techniques, the day may soon come when the premalignant potential of an oral dysplasia is judged not only by the degree of dysplasia, but also by the presence of molecular changes in the lesion. For example, our study showed that only 43% of mild dysplasia and 57% of moderate dysplasia exhibited any LOH. It is possible that those early premalignant lesions (mild and moderate dysplasia) with any LOH would have a higher risk of malignant transformation than those early premalignant lesions that do not demonstrate any allelic loss. Multiple chromosome loss was not seen in reactive lesions, but progressively increased with progression of the oral lesions. Mild and moderate dysplasia showed multiple chromosome loss at 19% and 38% of cases respectively. Again, it is possible that those early premalignant lesions that demonstrate multiple chromosome loss would have an even higher risk of progressing into carcinoma than those that only display single chromosome loss. Similarly, since LOH at 17p seemed to be a later event than LOH at 3p and 9p, it is also possible that early premalignant lesions that demonstrate LOH at 17p would have a greater risk than those premalignant lesions which demonstrate LOH at 3p and 9p.

While the frequency of LOH at 9p and 17p seems to increase steadily with progression of oral lesions, the frequency of LOH at 3p was similar among the premalignant lesions, but jumped with transformation of premalignant lesions into carcinomas. Since there were three presumptive tumour suppressor gene loci within 3p, it is possible that the transformation of a premalignant lesion into cancer is accompanied by LOH at a different loci at 3p, although further hit at the same common loci, such as *FHIT* loci, is possible. Further study is needed to study different loci loss during the multistage oral carcinogenesis.

10.2. LOH and Smoking

As mentioned earlier, the primary objective of the study was to investigate the LOH status in different diagnostic groups and to determine the relationship between LOH and progression of oral lesions to malignancy. However, one factor, the history of tobacco usage, was analyzed to

see its relationship to LOH. Carcinogens, such as those in tobacco, are known to affect the genetic pathways of carcinogenesis, and it would be of interest to see if LOH at certain chromosome arms are specifically related to use of tobacco in oral dysplasia and oral cancer.

p53 gene mutation and *p53* protein overexpression are strongly correlated with use of tobacco products and have been found in a high frequency in those cancers that are strongly related to th use of tobacco products, such as head and neck SCC, but in lower frequency in those cancers moderately related to the use of tobacco products, such as bladder cancer, and rare in those cancers that are not related to the use of tobacco products, such as sarcomas. The loss of a tumour suppressor gene function is frequently associated with mutation of the TSG at one allele and LOH at the other allele. Since *p53* gene mutation is strongly correlated with the use of tobacco products, we have expected to see LOH at 17p correlating with the use of tobacco products, although information on allelic loss and smoking is scanty. The results of the study showed that dysplastic and cancer lesions of smokers had a higher frequency of LOH at 17p (66%) as compared with that of non-smokers (43%). However, this difference is not statistically significant. Nonetheless, the lack of statistical difference could have resulted from the small number of cases with known smoking habits in this study, and further study is need to answer the question.

The *FHIT* gene (located at 3p) is a very new tumour suppressor gene first found in 1996 (Ohta *e al*), and its relationship to tobacco usage and its function remains unclear. Two other tumour suppressor genes are also believed to be present on 3p, but again, their identity and their relationship to tobacco usage remains unknown. The results from this study showed that LOH a 3p, similar to LOH at 17p, was found in higher frequency in smokers in dysplastic and cancer patients (52%) as compared with that of non-smokers (37.5%). Again, similar to LOH at 17p, the difference is not significant, although the small number of cases with known smoking history could have been responsible for the indifference. Further studies are needed. LOH at 9p, on the

other hand, showed a similar frequency among both smokers (57%) and non-smokers (60%) in the dysplastic and cancer lesions.

10.3. Is Oral Lichen Planus (OLP) a Premalignant Lesion?

Presence of epithelial dysplasia and the degree of the dysplasia in oral lesions, at present, are the most reliable parameters used in estimating malignant potential of oral premalignant lesions. The debate about whether OLP is premalignant may be summarized as two theories: Theory 1, OLP *per se* has a malignant potential, albeit small. Theory 2, OLP *per se* is not premalignant, and only when it, like normal oral mucosa, becomes dysplastic (lichenoid dysplasia), should one consider it premalignant. Since OLP is such a common oral disease, the issue as to its premalignant nature is very important. If the second theory is correct, professionals should then only treat lichenoid dysplasia as a premalignant lesion.

The results of this study showed LOH in only 6% of OLP, a percentage even lower than that in reactive oral lesions (14%). The study used epulides, trauma-induced fibroepithelial polyps, gingival hyperplasia, periodontitis, and mucocele as benign reactive lesions (negative control). The loss in both OLP and reactive lesions was limited to a single chromosome arm, mostly at 3p (5/6 cases) with 1 case at 9p. In contrast, a much higher frequency of LOH was noted in dysplastic and malignant lesions, and the loss frequently involved multiple chromosome arms. Even mild dysplasia, a lesion with small malignant potential, demonstrated 43% LOH, which is significantly higher than that of OLP ($p=0.0017$).

There were no unusual features noted in the 6 cases with LOH in OLP and oral reactive lesions. The clinical presentation of the 2 cases of OLP with LOH was classical for OLP. The histology of the 6 cases was again reviewed and no dysplasia was noted. The presence of LOH in these 6 cases was not associated with use of tobacco products: 4 were from patients who never used tobacco products (4/41 non-tobacco users in these two groups of lesions, i.e., 10%) and 2 were

from smokers (2/21 smokers, i.e., 10%). The 4 cases with a history of negative tobacco usage were confirmed by telephoning the patients.

These data do not support the theory that OLP has a malignant potential. The significance of the low frequency of single allelic loss at 3p or 9p found in OLP and oral reactive lesions remains a question. LOH at multiple arms or LOH at certain other chromosome arms such as 17p may be more significant indicators for malignant risk.

Several other studies have also investigated LOH in oral hyperplasia or normal oral mucosa using similar microsatellite markers (Califano *et al* 1996; Mao *et al* 1996; Roz *et al* 1996; Emilion *et al* 1996; El-Naggar *et al* 1995; van der Riet *et al* 1994). Two studies found no loss in these tissues (Emilion *et al* 1996; El-Naggar *et al* 1995), while two studies reported 31% of LOH in oral hyperplastic lesions (Califano *et al* 1996; Mao *et al* 1996). Oral hyperplastic lesions (histologically demonstrating thickening of the squamous epithelium) may be divided into two groups: One group consists of those lesions of obviously traumatic and inflammatory origin as selected in this study (e.g. epulides, polyps, gingivitis, and mucoceles), and this group of lesions are not regarded as premalignant. The other group consists of those lesions clinically present as leukoplakia without obvious trauma or inflammatory etiologies, and this group may be included in the histological tumour progression model and may be the very early (pre-dysplastic) changes of oral carcinogenesis. This latter group should conceivably demonstrate a higher frequency of LOH than the former group. The non-dysplastic hyperplastic lesions from Mao *et al* (1996) were leukoplakias, while those from the study by Califano *et al* (1996) may also contain leukoplakias. The differences in the selection of oral hyperplastic lesions may have partly contributed to the discrepancy between our study and the other two studies in regard to LOH frequency. In addition the study by Califano *et al* (1996) used more microsatellite markers, which may also partly account for the higher frequency of LOH.

Does OLP, under the influence of oral carcinogens, undergo malignant transformation through th dysplastic pathway just like normal oral mucosa? Our results showed that one patient (a smoker) with both OLP and lichenoid dysplasia demonstrated LOH only in lichenoid dysplasia. It seems logical to presume that this patient originally had only OLP (multiple lesions at different sites), and subsequently, one lesion, possibly under the influence of smoking, underwent dysplastic changes and LOH. Preliminary data from this lab with a small number of samples have demonstrated that lichenoid dysplasia contains a high frequency of LOH, similar to oral dysplasia (data not shown). Further study is needed to confirm LOH in lichenoid dysplasia. If the results show similar LOH patterns to those of oral dysplasia, it would support the hypothesis that at least some OLPs, similar to normal oral mucosa, undergo malignant transformation through the dysplastic pathway, and that the progression of lichenoid dysplasia is similar to oral dysplasia at the molecular level.

The possibility that OLP is premalignant cannot be totally ruled out, as it may undergo malignant transformation through different pathways from that of oral dysplasia. For example, a recent study has shown that salivary gland tumours display patterns of allelic loss that differ from many other tumours in the head and neck region (Johns *et al* 1996). Studies are currently underway in the lab to investigate LOH at other alleles in OLP.

The World Health Organization defines premalignancy as "a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart" (Holmstrup *et al* 1992). Can one still consider OLP a premalignant lesion without support from genetic studies? Apparently, even people who strongly believe that OLP *per se* is not a premalignant lesion agree that OLP may provide a fertile soil for carcinogens to work on, since it is an inflammatory lesion and has higher cell turnover which heightens the chances of genetic errors caused by a carcinogen. Does this feature of 'fertile soil' qualifies OLP as a premalignancy? If this is accepted, other trauma -induced oral lesions, such as denture-induced epulides, and chronic inflammatory lesions, such as periodontitis, should also be included as premalignant lesions. Apparently, these latter

lesions are not regarded as premalignancies. If OLP has an intrinsic tendency to become malignan
instead of just providing a fertile soil, it should conceivably contain genetic changes that predispose this lesion. The rapid development of molecular biology techniques should make the detection of these gene changes, if they exist, possible in the near future.

11. APPENDIX

Table 1. Clinical and Pathological Data

Sample # and diagnosis	Sex	Age (years)	Site	Tobacco use
7 LP	F	35	buccal mucosa	smoker
8 LP	F	48	gingiva	no
13 LP	M	35	buccal mucosa	smoker
14 LP	M	37	buccal mucosa	no
20 LP	F	50	lateral border of tongue	no
29 LP	F	61	buccal mucosa	no
30 LP	F	45	tongue	smoker
31 LP	M	39	tongue	smoker
35 LP	F	50	gingiva	no
36 LP	M	30	buccal mucosa	no
38 LP	F	43	buccal mucosa	no
39 LP	M	49	buccal mucosa	no
40 LP	F	26	buccal mucosa	no
41 LP	F	56	buccal mucosa	no
42 LP	F	67	buccal mucosa	no
46 LP	M	55	buccal mucosa	no
47 LP	M	47	gingiva	no
48 LP	M	53	gingiva	no
49 LP	F	55	buccal mucosa	no
50 LP	M	51	buccal mucosa	no
56 LP	M	46	buccal mucosa	no
70 LP	M	51	buccal mucosa	former smoker
73 LP	M	47	tongue	no
74 LP	F	44	tongue	N/A
75 LP	F	66	buccal mucosa	no
84 LP	F	67	buccal mucosa	smoker
85 LP	M	7	buccal mucosa	no
86 LP	F	58	buccal mucosa	no
87 LP	M	37	buccal mucosa	no
89 LP	F	28	facial gingiva	no
91 LP	F	47	buccal gingiva	no
93 LP	M	47	tongue	smoker
94 LP	F	61	right buccal mucosa	no
12 Mild dysplasia	M	54	tonsilar fauces	N/A
21Mild dysplasia	F	50	gingiva	N/A
26 Mild dysplasia	M	66	gingiva	smoker
51 Mild dysplasia	M	67	soft palate	N/A
55 Mild dysplasia	F	81	soft palate	smoker

60 Mild dysplasia	F	44	floor of mouth	smoker
64 Mild dysplasia	M	52	floor of mouth	smoker
88 Mild dysplasia	F	67	tongue	former smoker
92 Mild dysplasia	F	47	tongue	smoker
108 Mild dysplasia	F	50	floor of mouth	smoker
180 Mild dysplasia	M	34	buccal mucosa	smoker
184 Mild dysplasia	F	33	buccal sulcus	smoker
186 Mild dysplasia	F	31	floor of mouth	smoker
187 Mild dysplasia	F	41	soft palate	smoker
188 Mild dysplasia	?	79	floor of mouth	N/A
191 Mild dysplasia	F	58	soft palate	smoker
192 Mild dysplasia	M	52	soft palate	N/A
201 Mild dysplasia	F	52	tongue	no
206 Mild dysplasia	M	41	hard palate	smoker
207 Mild dysplasia	F	66	buccal mucosa	N/A
209 Mild dysplasia	M	44	tongue	no
22 Moderate dysplasia	M	71	soft palate	pipe smoker
25 Moderate dysplasia	F	53	tongue	smoker
27 Moderate dysplasia	F	75	gingiva	N/A
32 Moderate dysplasia	M	63	floor of mouth	smoker
34 Moderate dysplasia	M	38	tongue	N/A
62 Moderate dysplasia	F	57	tongue	smoker
66 Moderate dysplasia	M	42	buccal mucosa	no
71 Moderate dysplasia	M	53	tongue	N/A
76 Moderate dysplasia	M	64	soft palate	smoker
143 Moderate dysplasia	M	65	tonsil	smoker
160 Moderate dysplasia	M	77	lip	N/A
163 Moderate dysplasia	M	37	tongue	smoker
165 Moderate dysplasia	M	61	tonsilar fossa	smoker
167 Moderate dysplasia	M	74	buccal mucosa	N/A
168 Moderate dysplasia	F	72	buccal mucosa	N/A
169 Moderate dysplasia	M	69	tongue	N/A
176 Moderate dysplasia	M	24	soft palate	smoker
177 Moderate dysplasia	F	82	tongue	smoker
185 Moderate dysplasia	M	50	tongue	N/A
189 Moderate dysplasia	N/A	43	soft palate	N/A
190 Moderate dysplasia	M	49	tongue	smoker
4 Severe dysplasia	F	65	tongue	N/A
5 c.i.s.	M	58	tongue	N/A
6 severe dysplasia	F	68	floor of mouth	smoker
9 severe dysplasia	M	42	sublingual mucosa	smoker
15 severe dysplasia	M	65	tongue	smoker
17 severe dysplasia	M	42	floor of mouth	former smoker
19 severe dysplasia	M	76	floor of mouth	smoker
45 severe dysplasia	F	71	tongue	N/A

58 severe dysplasia	M	55	floor of mouth	smoker
62 severe dysplasia	F	58	tongue	former smoker
67 c.i.s.	M	62	tongue	smoker
79 severe dysplasia	F	60	tongue	N/A
81 severe dysplasia	N/A	N/A	N/A	N/A
83 c.i.s.	M	66	tongue	no
109 severe dysplasia	M	69	tongue	N/A
126 severe dysplasia	M	61	tongue	smoker
164 c.i.s.	F	79	floor of mouth	no
1 SCC	F	46	gingiva	N/A
2 SCC	F	58	gingiva	smoker
3 SCC	M	67	gingiva	smoker
10 SCC	F	29	tongue	no
43 SCC	M	66	tongue	smoker
59 SCC	M	63	buccal vestibule	smoker
61 SCC	M	31	tongue	smoker
65 SCC	F	60	tongue	smoker
80 SCC	M	77	floor of mouth	N/A
90 SCC	M	75	floor of mouth	smoker
110 SCC	F	39	tongue	no
112 SCC	M	84	tongue	no
113 SCC	F	30	gingiva	N/A
114 SCC	F	75	gingiva	smoker
115 SCC	M	72	floor of mouth	N/A
116 SCC	M	66	floor of mouth	N/A
118 SCC	M	68	gingiva	smoker
123 SCC	M	63	tongue	smoker
125 SCC	F	74	gingiva	no
162 SCC	F	56	tongue	smoker
166 SCC	F	81	tongue	no
173 SCC	M	75	tongue	N/A
174 SCC	M	37	tongue	former smoker
175 SCC	F	30	tongue	no
196 SCC	F	54	tongue	N/A
197 SCC	M	42	tongue	smoker
199 SCC	F	89	tongue	N/A
213 SCC	F	70	tongue	N/A
11 Hyperplasia	M	37	gingiva	no
101 Hyperplasia	F	41	tongue	no
102 Hyperplasia	F	24	gingiva	no
103 Hyperplasia	F	36	gingiva	no
111 Hyperplasia	F	30	gingiva	smoker
127 Hyperplasia	M	42	lip vestibule	smoker
129 Hyperplasia	F	N/A	hard palate	smoker
130 Hyperplasia	M	37	gingiva	smoker

131 Hyperplasia	M	14	tip of tongue	no
132 Hyperplasia	M	9	left upper lip	no
133 Hyperplasia	M	15	gingiva	no
134 Hyperplasia	M	11	lower lip	no
135 Hyperplasia	F	29	left lower lip	no
136 Hyperplasia	F	8	lower right lip	no
137 Hyperplasia	F	40	tongue	smoker
138 Hyperplasia	M	17	tongue	no
139 Hyperplasia	F	8	gingiva	no
140 Hyperplasia	F	52	hard palate	smoker
141 Hyperplasia	F	13	gingiva	no
142 Hyperplasia	M	8	hard palate	no
144 Hyperplasia	M	7	lower lip	no
145 Hyerplasia	F	68	gingiva	no
146 Hyperplasia	M	16	gingiva	no
149 Hyperplasia	M	20	buccal mucosa	N/A
150 Hyperplasia	M	32	tongue	no
151 Hyperplasia	M	76	buccal mucosa	smoker
152 Hyperplasia	F	38	hard palate	smoker
23 Hyperplasia	M	48	gingiva	no
28 Hyperplasia	F	51	buccal mucosa	no

N/A: data not available

12. BIBLIOGRAPHY

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

Adams, J.M. and Cory, S. Transgenic models of tumor development. Science, *254*: 1161-1167, 1991.

Adamson, R., Jones, A.S., and Field, J.K. Loss of heterozygosity studies on chromosome 17 in head and neck cancer using microsatellite markers. Oncogene, *9*: 2077-2082, 1994.

Ah-See, K.W., Cooke, T.G., Pickford, I.R., Soutar, D., and Balmain, A. An allelotype of squamous carcinoma of the head and neck using microsatellite markers. Cancer Research, *54*: 1617-1621, 1994.

Ahomadegbe, J.C., Barrois, M., Fogel, S., Le Bihan, M.L., Douc-Rasy, S., Duvillard, P., Armand, J.P., and Riou, G. High incidence of p53 alterations (mutation, deletion, overexpressior in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene, *10*: 1217-1227, 1995.

Andre, K., Schraub, S., Mercier, M., and Bontemps, P. Role of alcohol and tobacco in the aetiology of head and neck cancer: a case-control study in the doubs region of France. Oral Oncology, European Journal of Cancer, *31B*: 301-309, 1995.

Ankathil, R., Mathew, A., Joseph, F., and Nair, M.K. Is oral cancer susceptibility inherited? Report of five oral cancer families. Oral Oncology, European Journal of Cancer, *32B*: 63-67, 1996.

Baker, S.J., Markowitz, S., and Fearon, E.R. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science, *249*: 9121990.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K.V., Hamilton, S., and Vogelstein, B. p53 gene mutations occur in combinations with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Research, *50*: 7717-7722, 1990.

Banoczy, J. and Sugar, L. Longitudinal studies in oral leukoplakias. Journal of Oral Pathology, *1* 265-272, 1972.

Barbone, F., Franceschi, S., Talamini, R., Barzan, L., Franchin, G., Favero, A., and Carbone, A. A follow-up study of determinants of second tumor and metastasis among subjects with cancer of the oral cavity, pharynx, and larynx. Journal of Clinical Epidemiology, *49*: 367-372, 1996.

Beckstead, J.H. Histochemistry. *In*: I. Damjanov and J. Linder. (eds.), Anderson's Pathology 10th Ed., pp. 185-186. Missouri: Mosby-Year Book Inc., 1996.

Bedi, G.C., Westra, W.H., Gabrielson, E., Koch, W., and Sidransky, D. Multiple head and neck tumours: evidence for a common clonal origin. Cancer Research, *56*: 2484-2487, 1996.

Berg, J.W., Schottenfeld, D., and Ritter, F. Incidence of multple primary cancers. Cancers of the respiratory and upper digestive system as multiple primary cancers. Journal of the National Cancer Institute, *44*: 263-270, 1970.

Bishop, J.M. Molecular themes in oncogenesis. Cell, *64*: 235-248, 1991.

Boyle, J.O., Hakim, J., and Koch, W. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Research, *53*: 4477-4480, 1993.

Brandt, B., Vogt, U., Schlotter, C.M., Jackisch, C., Werkmeister, R., Thomas, M., von Eiff, U., Bosse, G., Assmann, G., and Zanker, K.S. Prognostic relevance of aberrations in the erbB oncogenes from breast, ovarian, oral and lung cancers: double-differential polymerase chain reaction (ddPCR) for clinical diagnosis. Gene, *159*: 35-42, 1995.

Bundgaard, T., Bentzen, S.M., and Sogaard, H. HIstological differentiation of oral squamous cell cancer in relation to tobacco smoking. Oral Oncology, European Journal of Cancer, *31B*: 118-121, 1995.

Caamano, J., Zhang, S.Y., Rosvold, E.A., Bauer, B., and Klein-Szanto, J.P. p53 alterations in human squamous cell carcinomas and carcinoma cell lines. American Journal of Pathology, *142*: 1131-1139, 1993.

Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S., Corio, R., Lee, D., Greenberg, B., Koch, W., and Sidransky, D. Genetic Progression Model for head and neck cancer: Implications for Field Cancerization. Cancer Research, *56*: 2488-2492, 1996.

Canman, C.E., Wolff, A.C., Chen, C., Fornace, A.J.J., and Kastan, M.B. The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia. Cancer Research, *54*: 5054-5058, 1994.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. Oncogenes and signal transduction. Cell, *64*: 281-302, 1991.

Cheng, J.Q., Jhanwar, S.C., Klein, W.M., Bell, D.W., Lee, W., Altomare, D.A., Nobori, T., Olopade, O.I., Buckler, A.J., and Testa, J.R. *p16* alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. Cancer Research, *54*: 5547-5551, 1994.

Chung, G.T.Y., Sundaresan, V., Hasleton, P., Rudd, R., Taylor, R., and Rabbitts, P.H. Sequential molecular genetic changes in lung cancer development. Oncogene, *11*: 2591-2598, 1995.

Chung, K.Y., Mukopadhyay, T., Kim, J., Casson, A., Ro, J.Y., Goepfert, H., Hong, W.K., and Roth, J.A. Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer Research, *53*: 1676-1683, 1993.

Cimprich, K.A., Shin, T.B., Keith, C.T., and Schreiber, S.L. cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. Proceedings of the National Academy of Sciences of the United States of America, *93*: 2850-2855, 1996.

Cruz, I.B.F., Snijders, P.J.F., Steenbergen, R.D.M., Meijer, C.J.L.M., Snow, G.B., Walboomers, J.M.M., and van der Waal, I. Age-dependence of human papillomavirus DNA presence in oral squamous cell carcinomas. Oral Oncology, European Journal of Cancer, *32B*: 55-62, 1996.

Diller, L., Kassel, J., and Nelson, C.E. p53 functions as a cell cycle control protein in osteosarcomas. Molecular Cell Biology, *10*: 57721990.

Duffey, D.C., Eversole, L.R., and Abenmayor, E. Oral lichen planus and its association with squamous cell carcinoma: an update on pathogenesis and treatment implications. Laryngoscope, *106*: 357-362, 1996.

Eisenberg, E. and Krutchkoff, D.J. The clinical-pathologic forum in oral pathology. Journal of Dental Education, *47*: 552-554, 1983.

Eisenberg, E. and Krutchkoff, D.J. Lichenoid lesions of oral mucosa. Oral Surgery, Oral Medicine, Oral Pathology, *73*: 699-704, 1992.

El-Naggar, A.K., Hurr, K., Batsakis, J.G., Luna, M.A., Goepfert, H., and Huff, V. Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous carcinoma. Cancer Research, *55*: 2656-2659, 1995.

Emilion, G., Langdon, J.D., Speight, P., and Partridge, M. Frequent gene deletions in potentially malignant oral lesions. British Journal of Cancer, *73*: 809-813, 1996.

Fang, F. and Newport, J.W. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. Cell, *66*: 731-742, 1991.

Fearon, E.R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61*: 759-767, 1990.

Field, J.K. Genomic instability in squamous cell carcinoma of the head and neck. Anticancer Research, *16*: 2421-2432, 1996.

Field, J.K., Kiaris, H., Risk, J.M., Tsiriyotis, C., Adamson, R., Zoumpourlis, V., Rowley, H., Taylor, K., Whittaker, J., Howard, P., Beirne, J.C., Gosney, J.R., Woolgar, J., Vaughan, E.D., Spandidos, D.A., and Jones, A.S. Allelotype of squamous cell carcinoma of the head and neck: fractional allele loss correlates with survival. British Journal of Cancer, *72*: 1180-1188, 1995.

Foulkes, W.D., Brunet, J.S., Kowalski, L.P., Narod, S.A., and Franco, E.L. Family history of cancer is a risk factor for squamous cell carcinoma of the head and neck in Brazil: A case-control study. International Journal of Cancer, *63*: 769-773, 1995.

Franceschi, S., Levi, F., and La Vecchia, C. Decline in 5-year survival rates for cancer of head and neck (Letter). Lancet, *340*: 471992.

Gallo, O. and Bianchi, S. p53 expression: a potential biomarker for risk of multiple primary malignancies in the upper aerodigestive tract. Oral Oncology, European Journal of Cancer, *31B*: 53-57, 1995.

Gallo, O., Bianchi, S., Giovannucci-Uzzielli, M.L., Santoro, R., Lenzi, S., Salimbeni, C., Abbruzzese, M., and Alajmo, E. p53 oncoprotein overexpression correlates with mutagen-induced chromosome fragility in head and neck cancer patients with multiple malignancies. British Journal of Cancer, *71*: 1008-1012, 1995.

Gerson, S.J. Oral Cancer. Critical Reviews in Oral Biology and Medicine, *1*: 153-166, 1990.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. Mutations in p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Research, *54*: 4855-4878, 1994.

Haughey, B.H., Von Hoff, D.D., Windle, B.E., Wahl, G.M., and Mock, P.M. c-myc oncogene copy number in squamous carcinoma of the head and neck. American Journal of Otolaryngology, *13*: 168-171, 1992.

Hinds, P., Finlay, C., and Levine, A.J. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. Journal of Virologyf, *63*: 7391989.

Hoffmann, D., Melikian, A., and Wynder, E. Scientific challenges in environmental carcinogenesis. Preventive Medicine, *25*: 14-22, 1996.

Hollstein, M., Sidransky, D., and Vogelstein, B. p53 mutations in human cancers. Science, *253*: 491991.

Holmstrup, P., Thorn, J.J., Rindum, J., and Pindborg, J.J. Malignant development of lichen planus-affected oral mucosa. Journal of Oral Pathology, *17*: 219-225, 1988.

Hou, L., Shi, D., Tu, S., Zhang, H., Hung, M., and Ling, D. Oral cancer progression and c-erbB-2/neu proto-oncogenes expression. Cancer Letters, *65*: 215-220, 1992.

Hsu, T.C. and Furlong, C. The role of ethanol in oncogenesis of the upper aerodigestive tract: inhibition of DNA repair. Anticancer Research, *11*: 1995-1998, 1991.

Hunter, T. and Pines, J. Cyclins and cancer. Cell, *66*: 1071-1074, 1991.

Huppi, K., Siwarski, D., Dosik, J., Michieli, P., Chedid, M., Reed, S., Mock, B., Givol, D., and Mushinski, J.F. Molecular cloning, sequencing, chromosomal localization and expression of mouse p21 (Waf1). Oncogene, *9*: 3017-3020, 1994.

Ishwad, C.S., Ferrell, R.E., Rossie, K.M., Appel, B.N., Johnson, J.T., Myers, E.N., Law, J.C., Srivastava, S., and Gollin, S.M. Microsatellite instability in oral cancer. International Journal of Cancer, *64*: 332-335, 1995.

Ishwad, C.S., Ferrell, R.E., Rossie, K.M., Appel, B.N., Johnson, J.T., Myers, E.N., Law, J.C., Srivastava, S., and Gollin, S.M. Loss of heterozygosity of the short arm of chromosomes 3 and 9 in oral cancer. International Journal of Cancer, *69*: 1-4, 1996.

Jazwinski, S.M. Longevity, genes, and aging. Science, *273*: 54-58, 1996.

Johns, M.M.I., Westra, W.H., Califano, J.A., Eisele, D., Koch, W.M., and Sidransky, D. Allelotype of salivary gland tumors. Cancer Research, *56*: 1151-1154, 1996.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, !., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S.I., Johnson, B.E., and Skolnick, M.H. A cell cycle regulator potentially involved in genesis of many tumor types. Science, *264*: 436-440, 1994.

Kaur, J., Srivastava, A., and Ralhan, R. Overexpression of p53 protein in betel and tobacco related human oral dysplasia and squamous cell carcinoma in india. International Journal of Cancer, *58*: 340-345, 1994.

Kiaris, H., Spandidos, D.A., Jones, A.S., Vaughan, E.D., and Field, J.K. Mutations, expression and genomic instability of the H-ras proto-oncogene in squamous cell carcinomas of the head and neck. British Journal of Cancer, *72*: 123-128, 1995.

Kim, S.K., Fan, Y., Papadimitrakopoulou, V., Clayman, G., Hittelman, W.N., Hong, W.K., Lotan, R., and Mao, L. DPC4, a candidate tumor suppressor gene, is altered infrequently in head and neck squamous cell carcinoma. Cancer Research, *56*: 2519-2521, 1996.

Kinoshita, I., Dosaka-Akita, H., Mishina, T., Akie, K., Nishi, M., Hiroumi, H., Hommura, F., and Kawakami, Y. Altered *p16*INK4 and retinoblastoma protein status in non-small cell lung cancer: potential synergistic effect with altered p53 protein on proliferative activity. Cancer Research, *56*: 5557-5562, 1996.

Kramer, I.R., Lucas, R.B., Pindborg, J.J., Sobin, L.H. Definition of leukoplakia and related lesions: An aid to studies on oral precancer (WHO collaborating centre for oral precancerous lesions). Oral Surgery, 46: 518-539, 1978.

Krutchkoff, D.J. and Eisenberg, E. Lichenoid dysplasia: A distinct histopathologic entity. Oral Surgery, Oral Medicine, Oral Pathology, *60*: 308-315, 1985.

Kuo, M., Jeng, J., Chiang, C., and Hahn, L. Mutations of Ki-ras oncogene codon 12 in betel quid chewing related human oral squamous cell carcinoma in Taiwan. Journal of Oral Pathology and Medicine, *23*: 70-74, 1994.

Lane, D.P. and Crawford, L.V. T antigen is bound to a host protein in SV40-transformed cells. Nature, *278*: 2611979.

Lazarus, P., Garewal, H.S., Sciubba, J., Zwiebel, N., Calagnotto, A., Fair, A., Schaefer, S., and Richie, J.P.J. A low incidence of p53 mutations in premalignant lesions of the oral cavity from non-tobacco users. International Journal of Cancer, *60*: 458-463, 1995.

Lese, C.M., Rossie, K.M., Appel, B.N., Reddy, J.K., Johnson, J.T., Myers, E.N., and Gollin, S.M. Visualization of INT2 and HST1 amplification in oral squamous cell carcinoma. Genes, Chromosomes, and Cancer, *12*: 288-295, 1995.

Levine, A.J., Perry, M.E., Chang, A., Silver, A., Dittmer, D., Wu, M., and Welsh, D. The 1993 Walter Hubert Lecture: The role of the p53 tumour-suppressor gene in tumorigenesis. British Journal of Cancer, *69*: 409-416, 1994.

Li, X., Lee, N.K., Ye, Y., Waber, P.G., Schweitzer, C., Cheng, Q., and Nisen, P.D. Allelic loss a chromosomes 3p, 8p, 13q, and 17p associated with poor prognosis in head and neck cancer. Journal of the National Cancer Institute, *86*: 1524-1529, 1994.

Licciardello, J.T., Spitz, M.R., and Hong, W.K. Multiple primary cancer in patients with cancer c the head and neck: second cancer of the head and neck, esophagus, and lung. Int. J. Radiat. Oncol. Biol. Phys. *17*: 467-476, 1989.

Lin, Y., Miyamoto, H., Fujinami, K., Uemura, H., Hosaka, M., Iwasaki, Y., and Kubota, Y. Telomerase activity in human bladder cancer. American Journal of Pathology, *168*: 437-440, 1996.

Lydiatt, W.M., Davidson, B.J., Shah, J., Schantz, S.P., and Chaganti, R.S.K. The relationship of loss of heterozygosity to tobacco exposure and early recurrence in head and neck squamous cell carcinoma. The American Journal of Surgery, *168*: 437-440, 1994.

Lydiatt, W.M., Davidson, B.J., Shah, J., Schantz, S.P., and Chaganti, R.S.K. The relationship of loss of heterozygosity to tobacco exposure and early recurrence in head and neck squamous cell carcinoma. American Journal of Pathology, *168*: 437-440, 1994.

Macfarlane, G.J., Boyle, P., and Scully, C. Oral cancer in Scotland: changing incidence and mortality. British Medical Journal, *305*: 1121-1123, 1992.

Mao, L., Fan, Y., Lotan, R., and Hong, W. Frequent abnormalities in *FHIT*, a candidate tumor suppressor gene, in head and neck cancer cell lines. Cancer Research, *56*: 5128-5131, 1996.

Mao, L., Lee, J.S., Fan, Y.H., Ro, J.Y., Batsakis, J.G., Lippman, S., Hittelman, W., and Hong, W.K. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nature Medicine, *2*: 682-685, 1996.

Mao, L. and Sidransky, D. Cancer screening based on genetic alterations in human tumors. Cancer Research, *54S*: 1939S-1940S, 1994.

McFarland, M., Abaza, N.A., El-Mofty, S. Mouth, Teeth, and Pharynx. *In*: I. Damjanov and J. Linder. (eds.), Anderson's Pathology 10th Ed., pp. 1563-1615. Missouri: Mosby-Year Book Inc., 1996.

Murti, P.R., Daftary, D.K., Bhonsle, R.B., Gupta, P.C., Mehta, F.S., and Pindborg, J.J. Malignan potential of oral lichen planus: observations in 722 patients from India. Journal of Oral Pathology, *15*: 71-77, 1986.

Nagatake, M., Takagi, Y., Osada, H., Uchida, K., Mitsudomi, T., Saji, S., Shimokata, K., and Takahashi, T. Somatic in vivo alterations of the DPC4 gene at 18q21 in human lung cancers. Cancer Research, *56*: 2718-2720, 1996.

Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nature Medicine, *2*: 1035-1037, 1996.

Nawroz, H., van der Riet, P., Hruban, R.H., Koch, W., Ruppert, J.M., and Sidransky, D. Allelotype of head and neck squamous cell carcinoma. Cancer Research, *54*: 1152-1155, 1994.

Nigro, J.M., Baker, S.J., and Preisinger, A.C. Mutations in the p53 gene occur in diverse human tumour types. Nature, *342*: 7051989.

Ohta, M., Inoue, H., Cotticelli, M.G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C.M., and Huebner, K. The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell, *84*: 587-597, 1996.

Park, N.H., Li, S.L., Xie, J.F., and Cherrick, H.M. In vitro and animal studies of the role of viruses in oral carcinogenesis. Oral Oncology, European Journal of Cancer, *28B*: 145-152, 1992.

Partridge, M., Emilion, G., and Langdon, J.D. LOH at 3p correlates with a poor survival in oral squamous cell carcinoma. British Journal of Cancer, *73*: 366-371, 1996.

Partridge, M., Kiguwa, S., and Langdon, J.D. Frequent deletion of chromosome 3p in oral squamous cell carcinoma. Oral Oncology, European Journal of Cancer, *30B*: 248-251, 1994.

Pastan, I. and Fitzgerald, D. Recombinant toxins for cancer treatment. Science, *254*: 1173-1177, 1991.

Paterson, I.C., Eveson, J.W., and Prime, S.S. Molecular changes in oral cancer may reflect aetiology and ethnic origin. Oral Oncol, European Journal of Cancer, *32B*: 150-153, 1996.

Pennisi, E. New gene forges link between fragile site and many cancers. Science, *272*: 6491996.

Pershouse, M.A., El-Naggar, A.K., Hurr, K., Lin, H., Yung, W.K.A., and Steck, P.A. Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma. Oncogene, *14*: 369-373, 1997.

Pindborg, J.J., Epidemiological studies of oral cancer. International Dental Journal, *27*: 172-178, 1977.

Raybaud-Diogene, H., Tetu, B., Morency, R., Fortin, A., and Monteil, R.A. p53 overexpression in head and neck squamous cell carcinoma: review of the literature. Oral Oncology, European Journal of Cancer, *32B*: 143-149, 1996.

Rich, A.M. and Reade, P.C. Nuclear morphometry in experimental oral mucosal carcinogenesis. Oral Oncology, European Journal of Cancer, *32B*: 169-175, 1996.

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

Rowley, H., Jones, A.S., and Field, J.K. Chromosome 18: a possible site for a tumour suppressor gene deletion in squamous cell carcinoma of the head and neck. Clinical Otolaryngology, *20*: 266-271, 1995.

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

Salem, G. Oral lichen planus among 4277 patients from Gizan, Saudi Arabia. Community Dental and Oral Epidemiology, *17*: 322-324, 1989.

Saranath, D., Bhoite, L.T., Deo, M.G., Tandle, A.T., D'Costa, J., Kolhapure, R.N., Govardhan, M.K., and Banerjee, K. Detection and cloning of potent transforming gene(s) from chewing tobacco-related human oral carcinomas. Oral Oncology, European Journal of Cancer, *30B*: 268-277, 1994.

Saranath, D., Bhoite, T., and Madhav, G.D. Molecular lesions in human oral cancer: The Indian scene. Oral Oncology, European Journal of Cancer, *29B*: 107-112, 1993.

Saranath, D., Panchal, R.G., Deo, M.G., Sanghvi, V., and Mehta, A.R. Restriction fragment length polymorphisms of the human N-myc gene in normal healthy individuals and oral cancer patients in India. Indian Journal of Biochemistry and Biophysics, *31*: 177-183, 1994.

Sato, S., Nakamura, Y., and Tsuchiya, E. Difference of allelotype between squamous cell carcinoma and adenocarcinoma of the lung. Cancer Research, *54*: 5652-5655, 1994.

Schantz, S.P. Head and neck oncology research (review) (23 refs). Current Opinion in Oncology, *6*: 265-271, 1994.

Scully, C. and Burkhardt, A. Tissue markers of potentially malignant human oral epithelial lesions. Journal of Oral Pathol Med, *22*: 246-256, 1993.

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

Shear, M. Erythroplasia of the mouth. International Dental Journal, *22*: 4601972.

Shin, D.M., Lee, J.S., Lippman, S.M., Lee, J.J., Tu, Z.N., Choi, G., Heyne, K., Shin, H.J.C., Goepfert, H., Hong, W.K., and Hittelman, W.N. p53 expression: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. Journal of the National Cancer Institute, *88*: 519-529, 1996.

Sidransky, D. Molecular screening - how long can we afford to wait? Journal of the National Cancer Institute, *86*: 955-956, 1994.

Sidransky, D., Boyle, J., Koch, W., and van der Riet, P. Oncogene mutations as intermediate markers. Journal of Cellular Biochemistry, Supp, *17F*: 184-187, 1993.

Sigurgeirsson, B. and Lindelof, B. Lichen Planus and Malignancy. Archives of Dermatology, *127*: 1684-1688, 1991.

Silverman, S.J., Gorsky, M., and Lozada-Nur, R. A prospective follow-up study of 570 patients with oral lichen planus: persistence, remission, and malignant association. Oral Surgery, Oral Medicine, Oral Pathology, *60*: 30-34, 1985.

Slaughter, D.P., Southwick, H.W., and Smejkal, W. "Field Cancerization" in oral stratified squamous epithelium. Cancer, *6*: 963-968, 1953.

Solomon, E., Borrow, J., and Goddard, A.D. Chromosome aberrations and cancer. Science, *254*: 1153-1160, 1991.

Sozzi, G., Alder, H., Tornielli, S., Corletto, V., Baffa, R., Veronese, M.L., Negrini, M., Pilotti, S., Pierotti, M.A., Huebner, K., and Croce, C.M. Aberrant *FHIT* transcripts in merkel cell carcinoma. Cancer Research, *56*: 2472-2474, 1996.

Uchida, T., Wada, C., Ishida, H., Wang, C., Egawa, S., Yokoyama, E., Kameya, T., and Koshiba, K. p53 mutations and prognosis in bladder tumors. Journal of Urology, *153*: 1097-1104, 1995.

Uhlmann, E.J., D'Sa-Eipper, C., Subramanian, T., Wagner, A.J., Hay, N., and Chinnadurai, G. Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. Cancer Research, *56*: 2506-2509, 1996.

Uzawa, K., Suzuki, H., Komiya, A., Nakanishi, H., Ogawara, K., Tanzawa, H., and Sato, K. Evidence for two distinct tumour-suppressor gene loci on the long arm of chromosome 11 in human oral cancer. International Journal of Cancer, *67*: 510-514, 1996.

Uzawa, K., Yoshida, H., Suzuki, H., Tanzawa, H., Shimazaki, J., Seino, S., and Sato, K. Abnormalities of the adenomatous polyposis coli gene in human oral squamous-cell carcinoma. International Journal of Cancer, *58*: 814-817, 1994.

Uzawa, N., Yoshida, M.A., Oshimura, M., and Ikeuchi, T. Suppression of tumorigenicity in three different cell lines of human oral squamous cell carcinoma by introduction of chromosome 3p via microcell-mediated chromosome transfer. Oncogene, *11*: 1997-2004, 1995.

van der Riet, P., Nawroz, H., Hruban, R.H., Corio, R., Tokino, C., Koch, W., and Sidransky, D. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Research, *54*: 1156-1158, 1994.

Varley, J.M., Thorncroft, M., McGown, G., Appleby, J., Kelsey, A.M., Tricker, K.J., Evans, D.G.R., and Birch, J.M. A detailed study of loss of heterozygosity on chromosome 17 in tumours from Li-Fraumeni patients carrying a mutation to the TP53 gene. Oncogene, *14*: 865-871, 1997.

Vokes, E.E., Weichselbaum, R.R., Lippman, S.M., and Hong, W.K. Head and neck cancer. New England Journal of Medicine, *328*: 184-194, 1993.

Waga, S., Hannon, G.J., Beach, D., and Stillman, B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature, *369*: 574-578, 1994.

Warnakulasuriya, K., Chang, S.E., and Johnson, N.W. Point mutations in the Ha-ras oncogene are detectable in formalin-fixed tissues of oral squamous cell carcinomas, but are infrequent in British cases. Journal of Oral Pathological Medicine, *21*: 225-229, 1992.

Weinberg, R.A. Tumor suppressor genes. Science, *254*: 1138-1146, 1991.

WHO collaborating centre for oral precancerous lesions. Definition of leukoplakia and related lesions: An aid to studies on oral precancer. Oral Surgery, *46*: 518-539, 1978.

Wu, C.L., Sloan, P., Read, A.P., Harris, R., and Thakker, N. Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma of the oral cavity. Cancer Research, *54*: 6484-648[illegible] 1994.

Wu, X., Dave, B.J., Jiang, H., Pathak, S., and Spitz, M.R. Lung carcinoma patients with a family history of cancer and lymphocyte primary chromosome 9 aberrations. Cancer, *79*: 1527-1532, 1997.

Xu, L., Chen, Y., Huvos, A.G., Zlotolow, I.M., Rettig, W., Old, L.J., and Garin, C., P. Overexpression of p53 protein in squamous cell carcinomas of head and neck without apparent gene mutations. Diagnostic Molecular Pathology, *3*: 83-92, 1994.

Yeudall, W.A., Paterson, I.C., Patel, V., and Prime, S.S. Presence of human papillomavirus sequences in tumour-derived human oral keratinocytes expressing mutant p53. Oral Oncology, European Journal of Cancer, *31B*: 136-143, 1995.

Yokoyama, J., Shiga, K., Sasano, H., Suzuki, M., and Takasaka, T. Abnormalities and the implication of retinoblastoma locus and its protein product in head and neck cancers. Anticancer Research, *16*: 641-644, 1996.

Yoo, G.H., Xu, H.J., Brennan, J.A., Westra, W., Hruban, R.H., Koch, W., Benedict, W.F., and Sidransky, D. Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromosome 13q in head and neck squamous cell carcinoma. Cancer Research, *54*: 4603-4606, 1994.

Zariwala, M., Schmid, S., Pfaltz, M., Ohgaki, H., Kleihues, P., and Schafer, R. p53 gene mutations in oropharyngeal carcinomas: a comparison of solitary and multiple primary tumours and lympynode metastases. International Journal of Cancer, *56*: 807-811, 1994.

Zhang, S., Klein-Szanto, A.J.P., Sauter, E.R., Shafarenko, M., Mitsunaga, S., Nobori, T., Carsor D.A., Ridge, J.A., and Goodrow, T.L. Higher frequency of alterations in the *p16*/CDKN2 gene ir

squamous cell carcinoma cell lines than in primary tumors of the head and neck. Cancer Research, *54*: 5050-5053, 1994.

Zhang, L., Rosin, M., Priddy, R., and Xiao, Y. p53 expression during multistage human oral carcinogenesis. International Journal of Oncology, *3*: 735-739, 1993.

Zhang, L., Mao, E.J., Xiao, Y., Priddy, R. and Rosin, M.P. p53 expression in oral lichen planus. Oncology Reports, *3*: 1145-1148, 1996.

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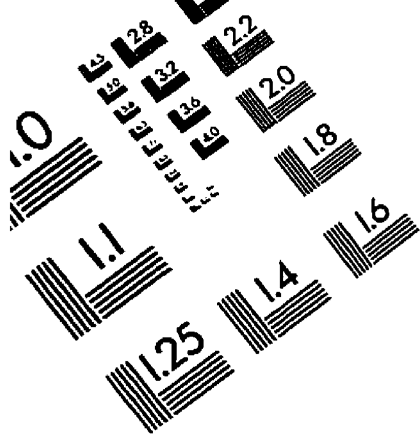

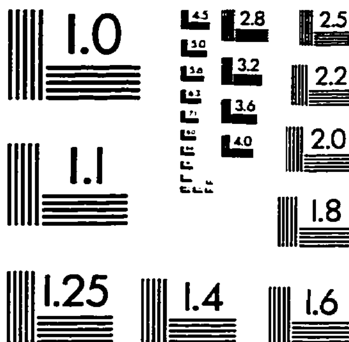

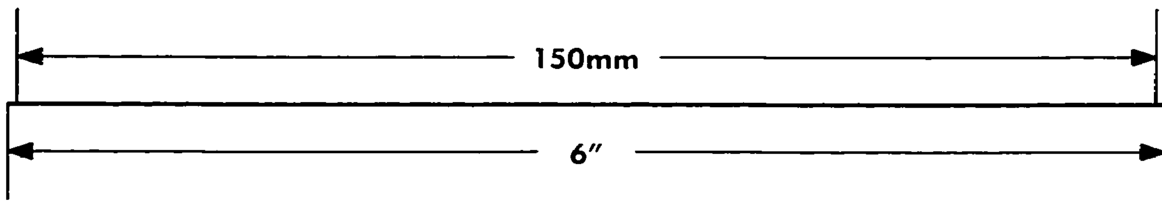

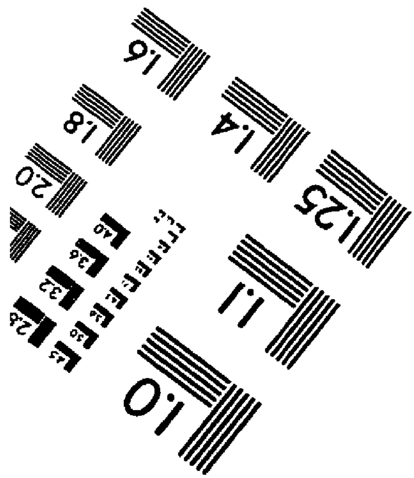

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