THE INVESTIGATION OF TOLUIDINE BLUE AND LOSS OF HETEROZYGOSITY PATTERNS IN ORAL PREMALIGNANT LESIONS

by

Hisae Nakamura

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NAME: Hisae Nakamura

DEGREE: Master of Science

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EXAMINING COMMITTEE:

Chair: Dr. Ted Milner

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

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

Dr. Amandio Vieira
Assistant Professor, School of Kinesiology
Simon Fraser University

Dr. Ravindra Shah
Associate Professor, Dentistry
Oral Biological and Medical Sciences
University of British Columbia
External Examiner

Date Approved: Dec 22, 2002

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Author:

(signature)

Hisae Nakamura

(name)

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ABSTRACT

Individuals with oral squamous cell carcinomas (SCCs) have a generally poor prognosis mainly due to late diagnosis of the disease. The identification of early stages of the disease (oral premalignant lesions, OPLs) is crucial to improving outcome. At present, prediction of outcome for OPLs relies on the histological assessment of biopsies for the presence and degree of dysplasia. Unfortunately, pathology is a poor predictor of outcome for the earliest, and most frequently observed OPLs.

Our recent study has shown that high-risk OPLs can be identified by assessing for loss of heterozygosity (LOH) using microsatellite analysis. Unfortunately, both molecular and histological assessments depend on the availability and accuracy of a biopsy. The determination of when and where to biopsy OPLs is a continuing challenge facing clinicians. In experienced hands, toluidine blue (TB) staining can be used to facilitate such determinations. However, at present it is not clear whether TB-positive OPLs differ in molecular alterations and cancer risk from negative lesions.

The objective of this thesis was to determine whether LOH profiles were different in TB positive and negative OPLs. A total of 67 OPLs were evaluated for TB staining, and assessed for LOH using 11 microsatellite markers on chromosome arms: 3p, 9p, and 17p. The main finding of the thesis was that TB-positive cases had a high-risk LOH than negative cases. They showed increased losses on multiple regions ($P = 0.004$), at 3p ($P = 0.04$), 9p ($P = 0.007$), and 17p ($P = 0.0001$). This positive association between TB staining and LOH

was independent of dysplasia. TB positive cases of hyperplasia or low-grade dysplasia showed higher frequencies of LOH at any loss ($P = 0.006$) and at 17p ($P = 0.005$).

In conclusion, these data show that patterns of allelic loss are different in TB-positive and TB-negative lesions with an increased proportion of cases showing patterns with an elevated risk of progression. The data support the use of this dye by clinicians to identify tissue that requires biopsy.

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ABBREVIATIONS

BCCA:	British Columbia Cancer Agency
CDK:	Cyclin-dependent kinase
CIS:	Carcinoma *in situ*
DNA:	Deoxyribonucleic acid
FOM:	Floor of mouth
H&E:	Hematoxylin and eosin
LOH:	Loss of heterozygosity
OPL:	Oral premalignant lesion
PC:	Phenol-chloroform
PCR:	Polymerase chain reaction
PEP:	Primer extention preamplification
TB:	Toluidine blue
TSG:	Tumor suppressor gene
UBC:	University of British Columbia
WHO:	World Health Organization

1. INTRODUCTION

1.1. Overview

Oral cancer is the 6th most common malignancy in the world. In Western countries, it accounts for up to 6% of cancers (Johnson, *et al.,* 1998). In Canada about 4,600 new cases of oral cancer arise every year, and 1,100 of these cases result in death (Rosati, 1993). In the United States approximately 30,000 new cases are diagnosed annually, and 8,000 people die each year (Wingo *et al.*, 1995). In comparison to the West, the rates of oral cancer are much higher in Southeast Asia, accounting for up to 40% of all cancers (Ishwad *et al.*, 1996). This high incidence is believed to be associated with the widespread practice of chewing tobacco in this region. In contrast, in Western countries, 80-90% of oral carcinomas are believed to be associated with the use of tobacco and alcohol.

Despite improvements in surgical, chemotherapeutic and radiation therapies, the 5-year survival rate for oral cancer has not improved in the last decade and has remained at about 50% (Silverman, 1981). This is one of the lowest survival rates among the major human cancers. This poor prognosis is believed to result from the detection of tumors at a late stage, the high recurrence rate of oral cancer and the increased incidence of second primary cancer. Current strategies to improve this dismal prognosis rely heavily on the identification and appropriate management of high-risk oral premalignant lesions (OPLs) before they progress into invasive cancer.

Although a number of clinicopathological characteristics are believed to have predictive value for risk of malignant transformation of OPLs, these criteria are far from adequate. In an effort to improve the ability to predict outcome for these lesions, clinicians have begun to incorporate molecular techniques into such evaluations. One such technique called microsatellite analysis, which will be used in this study, has already been shown to produce genetic profiles for OPLs that are strong predictors of outcome (Rosin et al., 2000). Unfortunately, both clinicopathological and molecular analyses suffer from a common limitation: they rely on the clinician making a decision to biopsy the lesion and on his/her ability to correctly localize the biopsy to the area of the lesion with the greatest risk of progression. Since localization of biopsies can be difficult based on clinical appearance alone, the development of new approaches to facilitate choice of the biopsy site is crucial.

Toluidine blue (TB) is a vital stain that has been used as a visual aid in the identification of oral cancer and high-grade precancer (Epstein and Scully, 1997). However, the mechanisms underlying the staining of tissue are not totally clear. As well, there is very little literature on the correlation between TB staining results and the genetic changes or cancer risks in OPLs. At present, it is not known why some OPLs stain with TB while others do not. Nor is it known whether genetic changes in TB-positive staining OPLs are different to negative staining lesions or whether the stain will identify those OPLs with a greater risk of progression. This thesis compared the molecular changes in positive and negative staining OPLs using microsatellite analysis for loss of heterozygosity (LOH) of loci on 3 chromosomal arms (3p, 9p and 17p). The objective of the thesis was to determine whether TB stain identifies lesions with a pattern of molecular change that has been associated with a

greater progression risk. If so, it would provide support for the use of this dye by clinicians to identify tissue that requires biopsy.

In the following sections, the histology of oral mucosa will be discussed along with a summary of clinicopathological and molecular indicators of oral cancer risk. In addition, the literature on the use of TB to predict outcome for cancer and precancerous lesions is also reviewed.

1.2. Histology of oral mucosa

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

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

The overlying stratified squamous epithelium is composed of four cell types; basal cells, prickle cells, granular cells, and cornified cells. The cuboidal-shaped basal cells form a very thin layer (usually single-cell layer) that exists between epithelium and connective tissue.

The cells within this basal cell layer are the only ones that have the capacity to divide and give rise to more new basal cells or differentiate into prickle cells (located in the middle part of the epithelium). As the cells mature, they migrate toward the surface, changing their shapes into more elongated and flattened forms. Once reaching the surface, they will eventually be desquamated.

Like many other parts of the body with mucosa lining such as esophagus and cervix, the majority of the oral cavity lining is not keratinized. However, some oral epithelium regions susceptible to mechanical forces such as gingiva and hard plate are covered with keratin and are referred to as masticatory mucosa (Wertz *et al.*, 1993). This mucosa serves as a very effective mechanical and permeability barrier. The dorsum of the tongue is composed of a specialized epithelium, that is a mixture of both non-keratinized and keratinized tissues which are attached tightly to the underlying tongue muscle (Wertz *et al.*, 1993). The non-keratinized oral regions such as buccal and the floor of the mouth are more flexible, thus accommodating the actions of chewing and speaking (Wertz *et al.*, 1993).

Over 90% of oral malignancies develop from this stratified squamous epithelial tissue, hence giving rise to the name, Squamous Cell Carcinoma (SCC).

1.3. Oral premalignant lesions

In 1978, the World Health Organization proposed the universal definition of a premalignant lesion as: "a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart". In the oral cavity, most premalignancy occurs in the form of a white patch, leukoplakia, which is defined as "a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion" (WHO, 1978). The less common clinical presentation of oral premalignant lesions is erythroplakia, defined as "a lesion that presents as a red area and cannot be diagnosed as any other definable lesion" (Pindborg *et al.*, 1996). As implied in the definition of the premalignancy, these lesions have increased likelihood of cancer risk; however, the majority of premalignant lesions will not become cancerous.

Currently our ability to predict which OPLs will become cancerous is limited. However, it is known that a number of clinicopathological features affect the malignant risk of oral premalignant lesions. These characteristics will be discussed in detail in the following section.

1.4. Clinicopathological risk factors and prediction of cancer risk of OPLs

1.4.1. Clinical appearance and cancer risk

The most significant clinical element that captures many investigators' and clinicians' interest is the presence of a "white patch" or leukoplakia. This is because the majority of oral premalignancies (80-90%) are composed of leukoplakia and people with leukoplakia are known to have a 5-fold elevation in risk of developing oral carcinoma in comparison to the general population (Waldron *et al.*, 1975; Shibuya *et al.*, 1986). Although leukoplakia are predominantly white, they sometimes appear yellow or light brown (WHO, 1978). They occur throughout the oral cavity; however, in Western countries the common sites (in descending order) are buccal mucosa, gingival, palate, tongue, the floor of the mouth and lip (Silverman *et al.*, 1984).

Leukoplakia can be classified into two major types. The most common form, called homogeneous leukoplakia, is predominantly white and has a smooth, sometimes slightly wrinkled texture. It is often found on buccal mucosa. The majority of these leukoplakia do not progress into cancer (Waldron *et al.*, 1975). The other type, referred to as non-homogeneous leukoplakia, has a rough surface with tiny wart-like projections and often has speckles of red patches. This latter form accounts for only 10% of all leukoplakia and has a much higher malignant transformation potential than homogeneous leukoplakia (Axell *et al.*, 1996).

The cause of leukoplakia remains a mystery. Possible etiological factors may be tobacco and trauma, which are known to induce hyperkeratosis. Interestingly, most leukoplakia also show characteristics of hyperkeratosis, which suggest a possible link to trauma or tobacco-related etiology. Also, some argue that there may be a correlation between leukoplakia and Vitamin A and B deficiencies (Ramaswamy *et al.*, 1996; Silverman *et al.*, 1996). This argument is still ongoing.

There is some evidence that leukoplakia progress clinically in stages. Lesions begin as thin, gray (or gray-white) plaques, sometimes fissured or wrinkled, with a translucent, flat appearance. Such early leukoplakia are known as homogeneous leukoplakia, thin leukoplakia or preleukoplakia (Bouquot and Whitaker, 1994). Late-stage leukoplakia share the same characteristics as non-homogeneous leukoplakia and, over time, they develop deepened fissures, pointed projections and red patches on the surface (Bouquot and Whitaker, 1994).

Many researchers have linked the non-homogeneousness of leukoplakia to a high cancer risk. For example, Rajendran *et al.* (1989) reported that 48% of all carcinomas had a verrucous characteristic (numerous pointed projections), which is one of the features of non-homogeneous lesion. Similarly, all 10 verrucous lesions in a study by Zakrzewska *et al.* (1996) showed evidence of carcinoma. In addition, Pindborg *et al.* (1968) reported that 7 of 11 leukoplakia patients who developed oral cancer between 1955 and 1964 displayed speckled leukoplakia, which is also classified as non-homogeneous leukoplakia. A similar correlation between speckled leukoplakia and carcinoma was reported by Shafer and

Waldron (1961). These studies suggest that there is a strong correlation between malignant risk and certain clinical features such as verrucous, speckled forms or non-homogenous leukoplakia. In fact, Bouquot and Whitaker (1994) suggest that leukoplakia progresses clinically into higher stages that have a more fissured, granular, and/or non-homogeneous appearance as well as erythroplakic patches, all of which are associated with increased cancer risk. It is of interest to note that cancer is currently viewed as a "genetic disease" (see section 1.5.4.) driven by the accumulation of specific genetic alterations. Thus, it can be further hypothesized that as a lesion progresses, it acquires more genetic alterations, which induce phenotypic changes. In other words, with genetic change, it is more likely for lesions to develop more fissures, projections and erythema, which define the characteristics of late-stage leukoplakias. This implies that the clinical features seen in the late phases are likely to possess more genetic alterations, thus they tend to have higher cancer risk than those in the early phases. More research is required in this area as there is little information on the correlation between clinical appearance and cancer progression.

Unfortunately the diagnostic criteria for both leukoplakia (white lesions) and erythroplakia (red lesions) are not solid. The diagnoses of leukoplakia and erythroplakia are frequently derived by exclusion because leukoplakia can be very hard to differentiate from reactive non-premalignant hyperplastic lesions (also presenting clinically as white lesions), and erythroplakia can be indistinguishable from inflammatory lesions (also presenting clinically as red lesions). In addition, the clinical features and appearance of leukoplakia frequently change over time and vary greatly among individuals as well as among lesions, adding to the

difficulty of diagnosis. It is extremely challenging to clinically isolate the leukoplakic lesions that are more likely to progress into cancer.

These difficulties in diagnosing and classifying leukoplakia (especially those with risk of progression) have led to a wide ranges in malignant transformation rates for these lesions, with values in the literature that span from a low of 0.13% to 31.4% (Leonardelli-Talamazzi, 1950; Pindborg *et al.*, 1968; Kramer *et al.,* 1970; Banoczy, 1977; Maeker-Burkhardt, 1978; Einhorn-Wersall, 1967; Silverman *et al.,* 1976; Roed-Petersen, 1971; Gupta *et al*, 1980; Bouquot *et al.*, 1988; Roz *et al.*, 1996; Schepman *et al.,* 1998; Lee *et al.*, 2000; see Table 1). This seeming discrepancy in the malignant transformation rates can be attributed to three main factors: 1) different follow-up times; 2) case-selected biases; and 3) variation in definitions of leukoplakia. Higher rates of malignant transformation tend to be associated longer follow-up times, since they give the lesion more time to develop into cancer. This was the case in a report made by Rosati in 1993 in which transformation rates were revised from old estimates of 2.2%-6% from a short follow-up time to recent estimates of 16.2%-17.5% from a longer follow-up time. A second major difference is whether or not leukoplakia include both homogeneous and non-homogenous lesions. Inclusion of homogeneous leukoplakia would result in a lowering of cancer risk. Finally, as previously mentioned, the definition of leukoplakia and its diagnosis tend to vary among researchers and clinicians. In fact, many clinicians diagnose white lesions derived from hyperkeratotic response to irritant or cheek biting as "leukoplakia". These lesions can be rubbed off or regress shortly after the irritant is removed, while "true leukoplakias" do not (Axell *et al.,*

1996). Inclusion of such reactive lesions in an analysis would lead to a decrease in the transformation rate.

Table 1. Malignant transformation in oral leukoplakia[1]

Author	Country	# of patients	Malignant transformation rate (%)
Silverman *et al.*, 1976	India	4762	0.13
Gupta *et al.*, 1980	India	360	0.3
Gupta *et al.*, 1980	India	410	2.2
Schepman *et al.*, 1998	Netherlands	166	2.9
Roed-Petersen, 1971	Denmark	331	3.6
Einhorn-Wersall, 1967	Sweden	782	4.0
Bouquot *et al.*, 1988	U.S.A.	463	10.3
Maeker-Burkhardt, 1978	Germany	200	12.0
Sturgis-Lund, 1934	U.S.A	143	13.0
Leonardelli-Talamazzi, 1950	Italy	268	19.8
Pindborg *et al.*, 1968	Denmark	248	4.4
Kramer *et al.*, 1970	England	187	4.8
Banoczy, 1977	Hungary	670	6.0
Silverman *et al.*, 1968	U.S.A.	117	6.0
Silverman *et al.*, 1984	U.S.A.	257	17.5
Lee *et al.*, 2000	U.S.A.	70	31.4

[1]Modified from Bouquot *et al.*, 1994 and Silverman *et al.*, 1984.

1.4.2. Size and duration of the lesion and cancer risk

Another clinicopathological factor that affects cancer risk is the size of the premalignant lesion. Generally, the bigger the premalignant lesion, the more likely it is to transform into cancer. Most premalignant lesions are small and either regress completely or decrease in size and never become cancerous. For example, Pindborg *et al.* (1968) examined 248 leukoplakia patients in a 9-year follow-up study. They reported that 20.1% of the leukoplakia disappeared completely and 17.8 % showed a reduction in size. Only 3.3% of all leukoplakia increased in size, and 3.7 % developed into carcinoma. Unfortunately, this study failed to report whether leukoplakia which later became carcinomas had increased in size. Nor was there any report of whether the initial size of a lesion was associated with clinical outcome (complete regression, partial regression, progression to cancer). Such additional information would be needed to confirm the correlation between size and cancer risk. Currently, the consensus is that small premalignant lesions and lesions that exhibit a reduction in size over time have a lower risk of malignant transformation than others that are larger in size. The "cutoff" for this size designation is not known.

Duration of a lesion is also felt to be predictive of outcome, with leukoplakia of long duration being associated with a higher risk of malignant transformation. This does not, however, imply that all leukoplakia with long duration will inevitably become cancerous. Normally, carcinoma with leukoplakia origin develops two to four years after the onset of a white plaque, but sometimes it occurs decades later (Bouquot *et al.*, 1988; Silverman *et al.*, 1984). Similarly, in other exceptional case, the malignant transformation occurs rather

quickly. In Leonardelli and Talamazzi's study in 1950, 49% of the patients developed cancer within three months after the initial check-up and 62% within one year. In comparison, Pindborg *et al.* (1968) found in their study that 36% of their subjects developed malignancy within one year, and 64% after one year. Of the 64 %, half developed cancer after 3 or 4 years. From the results obtained from early studies, it can be inferred that although there are some exceptions of rapidly occurring carcinomas, it usually takes a long time for a leukoplakia to transform into cancer, and older lesions are more likely to become cancerous than younger ones.

When studying the correlation between the duration of the lesion and its cancer risk it is desirable to consider a histological evaluation as well because cancer risk varies greatly, depending on the severity of dysplasia. Older lesions tend to have more severe dysplastic changes than younger ones because, simply, they have a longer time to acquire more DNA alterations, which lead to histological changes.

1.4.3. Site of the lesion and cancer risk

The high-risk sites in malignant transformation within the oral cavity are the floor of mouth (FOM), ventrolateral tongue, and soft palate-anterior-pillar-retromolar complex (soft-palate complex). There are variations in the percentage of malignant transformation among different regions of the mouth; however, these three sites generally show a much higher risk than other sites of the oral cavity. In fact, non-homogeneous leukoplakias with higher malignant potential are frequently observed on the FOM and tongue.

Some of the strongest evidence in support of this association between lesion site and cancer risk comes from 3 studies. In a study by Shafer (1980), 28% of high-grade OPLs (severe dysplasia and *CIS*) were found on the floor of mouth and 24% were on the tongue. High-grade lesions have a higher risk of malignant transformation (see section 1.4.6.). Mashberg *et al.* (1976) observed 97.5% of 222 cancer cases developing at high-risk sites. This result was later confirmed by the same research group in 1986 using 102 Italian patients. In the latter investigation, it was shown that 84% of cancers were present in the FOM, the ventral tongue, and the soft-palate complex. Finally, a large study conducted by Waldron *et al.* in 1975 with 3,256 cases of leukoplakias showed that dysplasia and carcinoma cases most commonly resided on the FOM (42.9%) and on the tongue (24.2%).

Though lesion site is known to be one of the major factors affecting cancer risk, no one has provided a satisfactory explanation for the divergence of malignant potential in different regions of the mouth. Many theories have been proposed, however. One such theory is that keratinization and heavy density of collagen might protect some tissue from carcinogens (Mashberg and Samit, 1989). Such characteristics can be seen in low-risk sites, especially in the cheek mucosa. In contrast, high-risk sites are usually covered by fragile, thin squamous epithelium with very little, if any, keratin. Also, their epithelial papilla is either absent or very short and their lamina propria is narrow, increasing cancer risk (Mashberg and Samit, 1989). A final hypothesis is that high-risk sites collect carcinogens more easily due to their anatomical structure and functions (saliva excretion) and thus have a prolonged exposure to carcinogens compared to low-risk sites (Jovanic *et al*, 1993). This would greatly increase

the probability of the lesion acquiring the critical mutations required for malignant transformation.

1.4.4. Etiology and cancer risk

Many etiological factors are believed to be associated with carcinogenesis. Tobacco is the most well-known and most influential risk factor involved in cancer development. The correlation of tobacco use and oral cancer, as well as lung cancer, has been investigated in great detail, and most of the findings confirm the etiologic role of tobacco in this disease. In Southeast Asia, particularly in India, the prevalence of chewing tobacco use is believed to be responsible for the high incidence of oral cancer. In contrast, cigarette smoking and drinking are thought to be the cause of this disease in the Western world. This combination of personal habits can increase the cancer risk by 3-15 times in the general population (Marshal *et al.*, 1992).

At least 2,000 chemicals have been identified in processed tobacco (Pershagen *et al.*, 1996). Some of those chemicals function as initiators and some as promoters in the carcinogenesis process. Thus, a longer duration of smoking is more likely to increase the risk of malignant transformation than shorter exposure. The long duration gives initiating and promoting chemicals enough time to induce damage to DNA in cells.

Another important risk factor related to oral cancer is alcohol consumption. Numerous studies have shown an increased oral cancer risk with drinking, and the risk tends to increase

in a dose-response fashion (Marshall *et al.*, 1992). Many recent studies focused on the combinational effect with smoking rather than alcohol effect only. When those two habits are practiced together, their cancer-initiating and promoting effects appear to be multiplied (Marshall *et al.*, 1992).

While known environmental carcinogens such as tobacco products are risk factors for oral cancer, people with leukoplakia without apparent known etiology (such as those who do not smoke and who are only social drinkers) in fact have a higher cancer risk than those with apparent etiology. It is generally presumed that these people are genetically susceptible, possessing defects in their ability to detoxify carcinogens or to repair carcinogen damage (Feigelson *et al.*, 1996; Hsu *et al.*, 1983). Consequently these people could develop premalignant or malignant lesions without exposure to a large quantity of carcinogens.

In summary, the most significant known etiological factors in oral cancer are carcinogens from tobacco and alcohol. Evidence of the relationship of such habits to oral cancer risk is extensive. It is believed that the cessation of either or both of these habits would reduce carcinogen exposure in the oral cavity and thus decrease one's susceptibility to the disease. Hence, smoking cessation counseling is a suggested intervention for individuals with premalignant lesions.

1.4.5. Gender, Candidiasis and cancer risk

Oral cancer is slightly more common in males than in females in the Western world (Silverman, 1994; Van der Waal *et al.*, 1997). This trend is most likely due to the harmful effect of tobacco and the fact that more males smoke than females. In Southeast Asia, this gender difference is even greater because of the high ratio of men with a tobacco chewing habit compared to women.

Candidiasis is a fungal infection; *Candida albicans* is the most common fungal species associated with oral leukoplakia (Krogh *et al.,* 1987). It is known that the majority of non-homogeneous leukoplakia is invaded by *Candida*. Since non-homogeneous leukoplakia is believed to have a higher malignant transformation potential, it has been suggested that candidiasis may play a causal role in cancer development (Krogh *et al.*, 1987). However, the mutagenicity and carcinogenicity of this fungus is still not well understood.

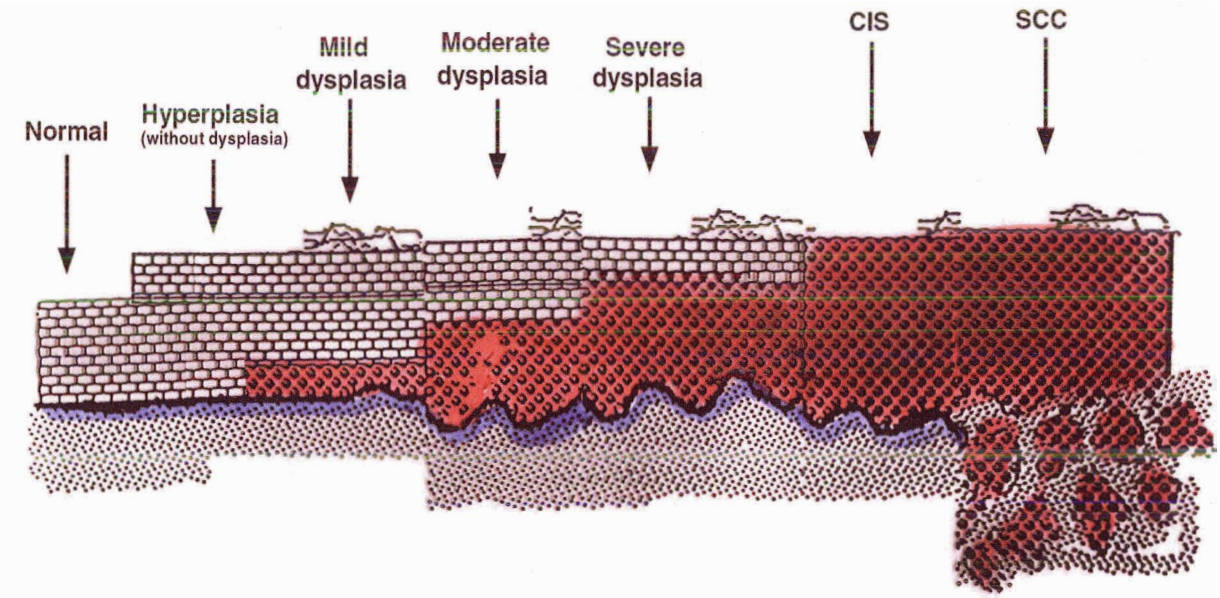
1.4.6. Histology and cancer risk

Currently, histological diagnosis is known as the gold standard for determining the cancer risk for premalignant lesions. This gold criterion requires that the clinician takes a biopsy from a suspicious premalignant lesion to be microscopically evaluated for the presence and degree of histological changes called "dysplasia". Dysplasia, as Lumerman describes it, "is the diagnostic term used to describe the histopathologic changes seen in a chronic, progressive, and premalignant disorder of the oral mucosa" (Lumerman *et al.*, 1995). It is

seen clinically as leukoplakia, erythroplakia, and leukoerythroplakia. It is also often observed in the margins of ulcers and in tissue adjacent to carcinomas (Lumerman *et al.*, 1995).

Pathologically, oral SCC is believed to develop through sequential stages of premalignancies, mild, moderate and severe dysplasia and carcinoma *in situ* (*CIS*) before becoming invasive SCC. The microscopic changes seen in all dysplastic cells include an increase in nuclear/cytoplasm ratio, dense nuclei, hyperchromatism, and change in shape of nuclei and cells. The degree of dysplasia is determined by how much the dysplastic cells are spread in the epithelium layer. For example, in mild dysplasia, the cytological and architectural changes are seen in 1/3 of the epithelium; in moderate dysplasia, such changes are seen in 2/3 of the epithelium; in severe dysplasia, it further spreads from 2/3 to the entire epithelium. It sometimes is challenging to distinguish carcinoma *in situ* (*CIS*) from severe dysplasia because in *CIS* the dysplastic changes are also observed throughout the entire epithelial layer. The dysplastic cells in *CIS* do not invade the underlying tissue at this stage. The invasion of the basal cell membrane and connective tissue occurs when the lesion gets to the stage of SCC. Once it penetrates through the underlying tissue, the cancer can spread through the lymphatic or circulatory systems to form a second tumor elsewhere.

Figure 1. Diagram showing different degrees of dysplasia



The degree and presence/absence of dysplasia are the critical keys in determining the cancer risk of premalignant lesions. For instance, leukoplakia showing dysplasia are considered to have a higher risk for malignant transformation than those without dysplasia. This high malignant transformation risk of leukoplakia with the presence of dysplasia has been reported by many investigators (Waldron and Shafer, 1975; Banoczy *et al.*, 1976; Lummerman *et al.*, 1995). The rate of malignant transformation is relatively high for dysplastic lesions. Epstein *et al.* (1996) reported that 43% of dysplastic leukoplakia progressed to malignancy. Another study conducted by Silverman *et al.* in 1984 found 36% of leukoplakia **with** dysplasia becoming cancerous after 7.2 years, whereas only 15% of leukoplakia **without** dysplasia became cancerous. As a rule of thumb, severe dysplasia and *CIS*, which are referred to as late-grade or late-stage dysplasia, have a much higher chance of progressing into carcinomas than milder dysplasias. Table 2 contains a summary of studies that report malignant transformation rates for high-grade dysplasia.

Table 2. Malignant transformation rates for late-stage OPLs[1]

Author(s)	Country	# Patients	Follow-up (years)	Malignant Transformation Rate (%)
Bouquot *et al.*, 1988[2]	U.S.A.	32[3]	10.8	15.6
Banoczy and Csiba, 1976	Hungary	23	6.3	21.8
Silverman, *et al.*, 1984	U.S.A.	22	7.4	36.0
Mincer, *et al.*, 1972	U.S.A.	16	3.0	18.8
Vedtofte, *et al.*, 1987	Denmark	14	3.9	35.7
Amagasa, *et al.*, 1985	Japan	12[3]	10.0	50.0
Lumerman *et al.*, 1995	U.S.A.	7	1.5	14.3
Average			**6.1**	**26.3**

(OPLs were histologically diagnosed to have either *CIS* or severe epithelial dysplasia.)
[1]Adapted from Bouquot *et al.*, 1994 and www.maxillofacialcenter.com/Table21.html.
[2]The only population-based study. Includes middle-class Caucasian cases only.
[3]Study includes only *CIS* cases

Histological progression models have been developed for oral cancer and are used to predict the progression of dysplasia as follows:

Hyperplasia without dysplasia→ Mild dysplasia → Moderate dysplasia→ Severe dysplasia→ Carcinoma In Situ → SCC.

This model has been used as a gold standard for predicting the cancer potential for many years, and it has performed well for high-grade premalignant cases. However, its predictive value is usually poor for low-grade premalignant lesions (i.e., hyperplasia and mild dysplasia). As mentioned earlier, only a small percentage of such lesions become cancerous, and the model has no way of predicting which of these low-grade lesions have the potential of becoming malignant. Therefore, a device or tool to detect those early lesions with cancer potential would be a breakthrough in cancer research.

1.4.7. History of head and neck cancer and further cancer risk

It is well known that when people have a history of aerodigestive tract cancers, they tend to have a high risk (10~30% times) of a second primary cancer or local/regional recurrence (Grant *et al.*, 1993). This poor prognosis is generally attributed to the microinvasion of malignant cells that were undetected initially and eventually emerged as recurrence at the former cancer site or spread to neighboring tissues as secondary cancers.

One of the problems facing clinicians who are following cancer patients after treatment is that leukoplakia in former oral cancer patients are harder to assess due to the mucosal changes induced by the primary treatment. For example, radiation therapy changes tissue appearance and decreases cellularity and vascularity, which predisposes to mucosal necrosis and ulceration. It also makes it harder for tissues to heal from benign lesions, injuries and insults (Epstein and Scully, 1997). If a biopsy were taken from such a lesion, the healing process would be further delayed. Therefore, physicians tend to be reluctant to perform biopsies in these patients, which results in a delay of the diagnosis (Epstein and Scully;

1997). Hence, any visual aids that would assist in detecting carcinomas and malignant margins would be in great demand in the medical field, as it would improve the predictive aspects of the survival rate and prognosis for oral cancer patients.

1.5. Critical genetic changes during oral cancer development

As mentioned earlier in this thesis, tumorigenesis is a multi-step event that takes place over a long period of time. The complex process of cancer development begins with one mutation at a critical control gene in a single cell, which brings about a growth advantage over its neighboring cells. Tumorigenesis takes place long after the initiation event; the tissue accumulates multiple critical gene mutations, which creates uncontrolled cell proliferation and selects specific mutated cells to expand into a genetic clone/tumor (Bishop *et al.*, 1991; Vogelstein *et al.*, 1992). Therefore, all daughter cells share the early genetic events, but subclones have additional genetic changes which give them a more aggressive growth advantage.

Ilyas *et al.* (1996) proposed that for a normal cell to become cancerous, a stepwise accumulation of genetic changes and a minimum number of such mutations are required to overcome the cellular growth control mechanism embedded in their normal genetic makeup. It is of great importance to delineate this process of alteration and the role it plays in oral cancer development, for it has the potential of revolutionizing the way in which premalignant lesions are managed, possibly allowing the differentiation of progressing lesions from non-progressive ones which are morphologically/clinically similar.

Two major types of genes are critical to tumorigenesis: proto-oncogenes and tumor suppressor genes (TSG). Each will be discussed in the following sections.

1.5.1. Oncogenes in oral premalignant and malignant lesions

During the development of cancer, normal cellular genes called proto-oncogenes become mutated to oncogenes through multiple mechanisms including point mutations, gene amplifications and chromosome translocation. Normal proto-oncogenes positively regulate cell division and differentiation. They code for proteins such as growth factors, growth factor receptors, protein kinases, signal transducers, and nuclear phosphoproteins. Mutation in or activation of such genes is often associated with increased cellular proliferation in addition to other critical alterations.

About fifty different oncogenes have been identified so far. The ones shown to be altered in head and neck SCC include *ras, cyclin-D1, c-myc, erbB-1, erbB-2, bcl-1, bcl-2, CK8, CK19,* and *int-2* (Kiaris *et al.*, 1995; Lese *et al.*, 1995; Saranath *et al.*, 1993).

Interestingly, different regions of the world show different frequencies of oncogene mutations. For instance, mutations of *ras* and *myc* are seen often in Eastern Asia, while in Western countries mutation of these genes is infrequent. This could be due to the frequently practiced and popular habits of chewing tobacco and betal quid in the East (Anderson *et al.*, 1994; Clark *et al.*, 1993).

1.5.2. Tumor suppressor genes (TSGs) in oral premalignant and malignant lesions

In contrast to proto-oncogenes, TSGs are negative regulators of cellular growth and differentiation, acting in a protective role. Among other functions, they act to suppress inappropriate cellular proliferation. In general, both copies of TSGs must be mutated for their protective protein function to be lost. Although multiple mechanisms exist for inactivation of the two alleles of a TSG, the classic example of the retinoblastoma gene, *Rb,* involves the point mutation of one allele followed by a loss of the second remaining allele (through multiple possible mechanisms including deletion, recombination, non-disjunction, etc.) (Knudson, 1971). More recently, the mechanisms inactivating dysregulated TSGs have been expanded to include epigenetic phenomena such as methylation. An example of a TSG inactivated by this mechanism is *p16,* also known as *MTS-1* (Major Tumor Suppressor-1) (Matsuda *et al.*, 1996; Merlo *et al.*, 1995; Papadimitrakopoulou *et al.,* 1997).

TSGs identified in head and neck SCC include *p53, Rb (*retinoblastoma*),* and *p16INK4A* (Gallo *et al.*, 1999; Jares *et al.*, 1999). Other genes are candidate TSGs; they are *FHIT* (fragile histidine triad), *APC* (adenomatous polyposis coli), *doc-1* (deleted in oral cancer), *VHL* (the gene responsible for von Hippel-Lidau syndrome) and *TBR-II* (the gene coding for transforming growth factor type II receptor) (Croce *et al.*, 1999; Mao *et al.*, 196; Uzawa *et al.*, 1994).

In normal cells, the well-balanced cellular proliferation activities are meticulously controlled by proto-oncogenes and TSGs. However, in the multi-step process of carcinogenesis, the

combination of oncogene gain and loss of TSGs results in abnormal, irregular growth of cells, which eventually lead to malignancy.

In oral carcinomas, TSG loss is observed as a common phenomenon, hence there have been many reports published on this matter. However, there are few studies of premalignant lesions. An efficient way to examine tissue for TSG alteration is to use a procedure called microsatellite analysis. This technique will be described in the following section.

1.5.3. Microsatellite analysis for loss of heterozygosity (LOH)

The study of genetic alteration in premalignant lesions is a crucial yet challenging endeavor. Such studies are limited by the difficulty in obtaining access to such biopsies (in comparison to biopsies of cancers) and by the small size of such samples, which limits the quantity of DNA that can be extracted from them. The microsatellite assay has been developed and modified to work with these minute DNA quantities. The rationale for the use of this assay is as follows.

Microsatellites are short tandem repeats of di, tri, or tetranucleotides such as GT GT GT GT, GTA GTA GTA, or GTAC GTAC GTAC. In humans, the number of such repeats varies greatly among different individuals, with the number of such repeats varying from 4~40 copies for different loci (Ah-See *et al.*, 1994).

The microsatellite assay makes use of these polymorphic regions (i.e. regions in which the maternal and paternal alleles are likely to have different numbers of copies of repeats) to screen for the loss of nearby TSGs in malignant and premalignant cells. Such loss, called loss of heterozygosity (LOH), can be very restricted to a small region around the polymorphic allele or extend to the whole chromosome. By choosing markers that map close to or within tumor suppressor genes, it is possible to quickly and efficiently gain information about deletion of TSGs. Indeed, this approach has been used often in the past to identify new TSGs in loci that are frequently and consistently lost in cancers such as the *Rb* gene, *MEN1*, and *APC* (Ah-See *et al.*, 1994; Fearon *et al.*, 1997).

Figure 2. Schematic view of loss of heterozygosity

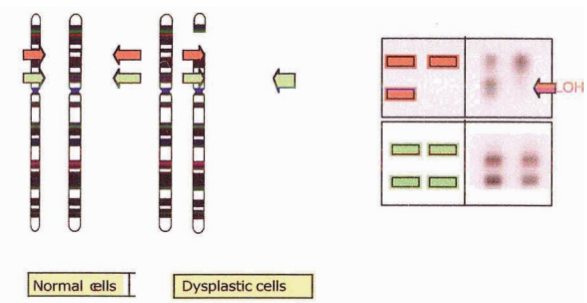


Figure 2 shows a classic example of loss of heterozygosity in abnormal cells. The colored arrows and bands represent different sets of alleles. In normal cells, both red and green sets of alleles are intact, while in dysplastic cells, both copies of green alleles are retained; however, one of the red alleles is lost, showing LOH. This loss may indicate that a closely situated putative TSG is also lost.

The value of this procedure lies in the numerous microsatellite markers available, well distributed throughout the human genome, present on average every 30-60 kilobase pairs (Ah-See *et al.*, 1994). Many of these regions are highly polymorphic in the population. Also, microsatellite analysis is far more sensitive than direct mutation analysis in genes because it requires a very small quantity of DNA, only 5 ng per reaction. In addition, it is possible to study loss of regions containing putative suppressor genes even before such genes have been identified and sequenced. This is the case for many of the chromosome regions thought to contain TSGs that have yet to be discovered (see the following section).

1.5.4. Molecular progression model for oral cancer development

It is now firmly established that carcinogenesis involves the alteration of multiple genes in a tissue. Over the last decade, there has been a fusion of this genetic information with histological studies to produce models for cancer progression at the genetic level.

In the late 1980s, Fearon and Volgelstein summarized the events that were known to take place during colon carcinogenesis and produced a molecular progression model for that disease. This model links the histological changes that take place during tumorigenesis to specific gene mutations. It suggests that a certain number of mutations and the accumulation of such changes must occur for a tumor to progress. At least seven genetic mutations have been identified as important to this model. They include *APC, K-ras, DCC, NF1, GAP, p53,* and *NM23* (Midgley *et al.*, 2000). It is believed that, generally, the genetic

alterations in colon cancer occur in this sequence; however, this order does not guarantee a cancer progression in this disease.

In comparison, the results from other studies suggest the accumulation of at least 6-11 genetic alterations is necessary for head and neck cancers (Emilion *et al.,* 1996; Renan, 1993). Califano *et al.* conducted a study in 1996 to establish a genetic progression model for head and neck cancers. Their samples included the whole spectrum of histologies: hyperplasia, dysplasia, *CIS*, and SCC. In the proposed model, they suggested a frequent pattern in the sequence of alterations that occurred in association with the progression of this disease. They noted that LOH at 9p was the earliest event, occurring in hyperplasia; that 3p and 17p were associated with dysplasia; and that 4q, 6p, 8p, 11q, 13q, 14q represented losses more frequently observed in *CIS* and SCC. The limitation of this study was that all dysplasias (mild, moderate, and severe) were categorized into one group. This creates a problem, since not only are they histologically different but they may also be genetically different.

1.5.5. LOH pattern predicts cancer risk of oral premalignant lesions

The prediction of cancer risk in precancerous lesions has always been a hot topic of debate. With the newly arrived efficient method of microsatellite analysis, the focus of research has shifted towards the investigation of different LOH patterns among early lesions.

The well-noted study of Mao *et al.* in 1996 examined LOH patterns of their leukoplakia patients in the hope of discovering a correlation between certain LOHs and cancer risk. They found that LOH at 9p21 and/or 3p14 increased the risk of malignant transformation in leukoplakia. Their report showed that **37%** of lesions **with LOH** progressed into cancer, while only **6%** of lesions **without LOH** showed progression. Similarly, a study by Partridge *et al.* (2000) showed that 94% of hyperplasia and dysplasia lesions with 9p and/or 3p losses progressed into SCC.

In order to acquire a broader knowledge of cancer progression in premalignant lesions, additional chromosome regions must be taken into consideration. In one of the studies conducted by our laboratory, Rosin *et al.* (2000) examined LOH at 3p, 4q, 8p, 9p, 11q, 13q, and 17p in hyperplasia, mild, and moderate dysplasias. Out of 116 patients without any history of cancer, 29 progressed into SCC. Different LOH patterns were observed in the progressing and non-progressing groups. The progressing group acquired more LOH than its counterpart. 97% of the cases in this group exhibited loss at 9p and/or 3p, with additional losses noted at higher frequency on other arms. Using the data generated in this study, a model was proposed which placed individuals with premalignant lesions into 3 categories of risk depending on LOH patterns: 1) low risk: retention at 3p and 9p; 2) intermediate risk: loss at 3p and/or 9p; and 3) high risk: loss at 3p and/or 9p plus 4q, 8p, 11q, 13q, or 17 p. The highest risk group (group 3) had a 33-fold increase in cancer risk compared to the low risk group (group 1). The study also suggests that for a premalignant lesion to progress, at least 3p and/or 9p LOH is necessary and for malignant transformation to occur, further additional loss must be present on other arms.

In summary, as can be seen from the aforementioned studies, LOH stands as an excellent marker for cancer prediction since it can differentiate the clinically and histologically similar progressing lesions from those non-progressing ones with low risk.

1.5.6. LOH and oral cancer

In this thesis, special attention is given to 3p and 9p losses as they are the regions most frequently lost in oral carcinomas and thus may be important risk indicators. In addition, LOH at 17p will be documented because *p53* is known to play a key role in cancer development. The additional LOH in the other 4 arms will be discussed as well. The following will describe the genes on these seven chromosomes.

Chromosome 3

There have been many reports of loss on chromosome 3 in head and neck cancers. The loss is most frequently seen at 3p13-21.1, 3p21.3-23, and 3p24-25 (Scully *et al.*, 1996; Patridge *et al.*, 1996; Roz *et al.*, 1996). Each of the three regions is presumed to contain at least one putative TSG.

Recently, a region of LOH at 3p14.2 has received much attention. It contains a locus known as "Fragile Site" or "FRA3B", whose name was derived from the fact that this site is very weak and breaks easily. This site makes an easy target for carcinogens found in tobacco to cause damage to the gene. Within FRA3B, a recently discovered gene called *FHIT* (Fragile Histidine Triad) exists and is believed to be altered in cancers of the esophagus, stomach,

colon, breast, cervix, lung, and head and neck (Pennisi *et al.,* 1996; Sozzi *et al.*, 1996). *FHIT* gene encodes a protein similar to the *Schizosaccharomyces Pombe* enzyme, diadenosine 5', 5'''-p1, p4-tetraphosphate (AP4A) assymetrical hydrolase. This protein cleaves AP4A into ADP & AMP. In theory, without normal control, diadenosine tetraphosphate would accumulate, leading to deregulated DNA synthesis and cell replication (Mao *et al.*, 1996).

In addition to the gene mentioned above, there is another recently identified gene called *VHL* at 3p24-25. It encodes a glycan-anchored membrane protein that functions in signal transduction and cell adhesion (Waber *et al.*, 1996). Genetic alterations in this gene have been observed in *VHL*-associated cancers. Some investigators suggest that the *VHL* gene may be involved in head and neck cancers (Uzawa *et al.*, 1998). However, the evidence in support of this idea does not favor such speculation (Uzawa *et al.*, 1998; Waber *et al.*, 1996). Mutations of this particular gene have not been identified nor has any kind of inactivation of the gene been reported in oral carcinomas. It can be speculated that it is the loss surrounding the *VHL* locus, not the gene itself, that occurs in carcinogenesis.

The possible TSGs at 3p24 and the other region, 3p21.3, are still under investigation.

Chromosome 4

LOH on chromosome 4 has been studied in cancers of many organs including liver, bladder, ovary and cervix (Pershouse *et al.*, 1997; Califano *et al.*, 1996). It is believed that the putative TSG may reside in a locus very close to the epidermal growth factor (*EGF)* locus at

4q25. There have been reports indicating that the loss at 4q25 is observed in the majority (75%) of head and neck cancers (Pershouse *et al.*, 1997). Loss at 4q24-26 is less frequently seen in this cancer (present in 47% of cases) and another putative TSG is thought to localize within this region (Califano *et al.*, 1996).

Chromosome 8

LOH at 8p has been reported to be relatively high in head and neck cancer with frequencies varying between 31% and 67% of cases (Ah-See *et al.*, 1994; Bockmuhl *et al.*, 1996; Califano *et al.*, 1996; el-Naggar *et al.*, 1995; Li *et al.*, 1994). Three discrete regions on this chromosome arm have been defined by deletion mapping of oral SCC: 8p23, 8p22, and 8p12-p21 (el-Nagger *et al.,* 1995). The evidence from previous studies suggests that LOH at 8p may indicate a higher clinical stage and poor prognosis for SCC (Li *et al.*, 1994).

Chromosome 9

LOH in 9p is known to be one of the most common losses in head and neck cancers occurring in more than 72% of cases (Scully *et al.*, 1996; Nawroz *et al.*, 1994). The most frequently lost sites on this chromosomal arm are 9p21-22 and 9p22-q 23.3 (Scully *et al.*, 1996; Mao *et al.,* 1996). Many reports have indicated 9p LOH in both SCC and in premalignant lesions; this suggests that this type of loss may be an early event in the course of carcinogenesis. One candidate TSG is *p16,* also called *MTS-1* (Major Tumor Suppressor-1), situated at 9p21. The function of *p16* is to control the cell cycle transition at G1-S phase by encoding a protein that binds to and inactivates cyclin-dependent kinases (CDK) 4 & 6, thus preventing phosphorylation of Rb protein and blocking cell cycle progression (Reed *et*

al., 1996). Therefore, *p16* loss may be the key in triggering unregulated cellular proliferation, thus leading to cancer progression in human. The information gathered on p16 protein inactivation from previous studies suggests that this gene could be the major TSG in oral cancer, especially in the early stage of the cancer development. In fact, many reports have indicated that this particular gene is inactivated by point mutation, deletion, or methylation in head and neck cancers. For example, Reed and Papadimitrakopoulou's investigation reported that 80% of their head and neck carcinomas and premalignant lesions had *p16* inactivated at the protein and/or DNA level.

Another TSG candidate on this chromosome is *p15*, which is situated close to the *p16* gene (El-Naggar *et al.*, 1995). In comparison to *p16,* whose product functions in the intracellular growth regulatory pathways, *p15* encodes a CDK inhibitor (for cyclin D-dependent kinases 4 & 6), which acts as an effector of extracellular growth inhibitory signals (Papadimitrakopoulou *et al.*, 1997). Further research is required to confirm the role of *p15* in this cancer.

Chromosome 11

There have been many reports indicating an association between LOH at 11q and a variety of cancers. The incidence of this type of loss in head and neck cancers varies between 39% and 61% (Bockhl *et al.*, 1996; Califano *et al.*, 1996; el-Nagger *et al.*, 1995; Uzawa *et al.*, 1996). The site that is frequently lost is 11q13 (Nawroz *et al.*, 1994). This region includes several proto-oncogenes, such as *INT2, bcl-1, Cyclin D1*, and *FGF*. Information from several studies suggests that the LOH at 11q13 may actually represent an amplification of

these genes rather than a deletion (Nawroz *et al.*, 1994). The allelic imbalance observed with the microsatellite assay does not allow one to differentiate between amplification and deletion of a chromosomal region. Both would alter the relative quantities of polymorphic alleles and result in a LOH. Some studies have compared data from fluorescent *in situ* hybridization (FISH) to microsatellite analysis at 11q13 and have shown a correlation. These data supports an amplification event as altering the LOH pattern in this region.

Amplification at 11q has been correlated with a poor prognosis for head and neck cancer (Papadimitrakopoulou *et al.*, 1997). In addition to 11q13, another site at 11q23 is considered to be a hot spot for a high risk of recurrence in head and neck cancers (Lazar *et al.*, 1998). More research is required to investigate this particular site.

Chromosome 13

More than half (52-67%) of head and neck cancers show LOH at 13q near the *RB* (retinoblastoma) locus, but not at the *RB* gene itself (Bockmuhl *et al.*, 1996; Califano *et al.*, 1996; Nawroz *et al.*, 1994). There is a strong correlation between LOH at 13q14.3 and lymph node metastasis for oral cancer and esophageal SCC (Ogawa *et al.*, 1998). In addition, another site in 13q21 has been investigated for the presence of a putative gene in this region.

Chromosome 17

Loss at 17p is seen in approximately 50% of head and neck cancers, most commonly at 17p13 and 17p11.1-12 (Scully *et al.*, 1996). One of the most studied TSGs, *p53,* is located at 17p13. This gene is frequently mutated and occurs in approximately 50% of all cancers; hence, it is known to be the gene with the highest frequency of mutations in human malignancies (Soussi, 1996). As expected, *p53* is one of the most common genetic alterations in oral cancer (Lazarus *et al.*, 1995). Its protein is involved in transcription activation, DNA repair, apoptosis, and G1 and G2 cell cycle inhibition after DNA damage (Nawroz *et al.,* 1994; Scully *et al.*, 1996). In addition to 17p13, accumulating evidence suggests that another locus known as CHRNB1 may contain a putative TSG at 17p11.1-12 (el-Nagger *et al.*, 1995).

Unlike other losses, such as 3p and 9p, 17p loss is believed to be a later event because it rarely occurs in low-grade lesions such as hyperplasia or mild dysplasia. For instance, studies conducted by Califano, Rosin, and Partridge *et al.* reported 3p and 9p LOHs as early events that occurred in early-stage premalignant lesions. In contrast, 17p loss as well as other losses at 4q, 6p, 8p, 11q, 13q, and 14q were reported as later events, occurring in late-stage premalignant lesions (*CIS* and SCC) (Califano *et al.*, 1996; Rosin *et al.*, 2000; Partridge *et al.*, 2000).

Two studies have compared the frequencies of LOH at 3p, 9p and 17p in oral premalignant lesions that later progressed to cancer to cases that did not (Table 3). LOH at these arms

was more frequently observed among the progressing cases (Rosin *et al.*, 2000; Partridge *et al.*, 2000).

Table 3. 3p, 9p and 17p LOH in progressing and non-progressing OPLs

Lesion Type	Progressive lesions	Non-progressive lesions	P value[3]
3p &/ or 9p LOH	96%[1] / 94%[2]	42%[1] / 27%[2]	**<0.0001**
17p LOH	39%[1] / 43%[2]	13%[1] / 8%[2]	**0.0064**

[1]LOH frequency from Rosin *et al.* (2000).
[2]LOH frequency from Partridge *et al.* (2000).
[3]Comparison of LOH between progressive and non-progressive lesions from Rosin *et al.* (2000).

1.6. The need for visual aid in clinical examination

Carcinoma of the oral cavity has a poor prognosis and survival rate compared to many other cancers, despite improvements in technology and molecular techniques. Part of the problem may arise from having the clinical assessment of lesions as the only identification tool for this disease. As stated earlier (section I.3), the majority of premalignant lesions are leukoplakia and most of them do not transform into cancer. Most often, it is very difficult to separate those with a high risk of transformation from those without because of the clinical similarities. Also, premalignant lesions are often mistaken for benign frictional hyperplasia or inflammation and sometimes premalignant and malignant lesions show no obvious clinical changes, adding to the difficulty of identification.

Biopsy is very crucial in improving prognosis because the histological evaluation of lesions relies on it. However, it can be quite challenging to take a biopsy, especially when the

lesion has characteristics of both erythroplakia and leukoplakia. In such a lesion, called leukoplakic erythroplakia, the colors tend to diffuse together, making it difficult to determine a precise biopsy site. Moreover, in a patient with a history of cancer, it is hard to assess and biopsy due to mucosal changes/damages induced by primary treatment. Clinicians are often hesitant to take a biopsy from such a region because the lesions take longer to heal. In order to avoid unnecessary biopsies, a visual aid is needed to assist in determining a good biopsy site.

1.6.1. PAP smear, a successful visual aid in screening for premalignant and malignant lesions of uterine cervix

There are two forms of cancer prevention. Primary prevention is, basically, the elimination of damaging habits such as alcohol and tobacco use, and secondary prevention is mainly the screening of high-risk populations such as smokers and people with cancer history. The majority of scientific attention is placed on the latter form of prevention. Medical and technological advances have created many new screening procedures or ways to identify high-risk premalignant lesions. In order for such procedures to be effective and accepted by public, they must be cost-effective, non-invasive and easy to perform.

One of the many examples of those procedures is the PAP (Papanicolaou) smear. It is a simple yet very effective screening procedure for cervical cancer that has been practiced for 55 years in North America (Casper *et al.*, 1998). This well-designed technique was discovered by a physician, George Papanicolaou, in the late 1940s, and has since decreased

the morbidity and mortality of cervical cancer tremendously. Before the arrival of this screening system, cervical cancer was known as the number one killer among women in the West. Not only in North America but also in Europe, the PAP smear has had a renowned effect. For example, it was introduced in Denmark and Finland in the late 1960s, but not in Norway. The mortality rate of cervical cancer has declined in the former two countries but has remained high in the latter country (Brewer *et al.*, 1994).

Recently, Omar *et al.* (2000) examined women from West Virginia University for abnormal cervical cells using PAP smears. In this procedure, a physician scrapes the lining of the cervix to examine for cytology. When abnormal cells are detected they are marked as high risk for cancer transformation, and patients are scheduled for another PAP smear test. In Omar *et al's* study, they found 24 % of the subjects who underwent the procedure showed abnormal cells indicating a high risk for cervical cancer. Similarly, Arora *et al.* (2001) found in their study that 10.5 % of the subjects showed abnormality between the period of 1994 and 1996. As can be seen from all these studies, the PAP smear continues to uncover cells having the potential of malignant transformation that otherwise might go undetected.

In comparison to the PAP smear and cervical cancer, oral cancer lacks an effective screening system. With oral precancerous lesions, the use of exfoliative cells for assessment is not suitable because these lesions tend to be keratinized, and exfoliative samples give a high frequency of false negative rate. Thus, the identification of aerodigestive tract cancer so far has been based solely on visual signs. This may directly affect the survival rate associated with oral cancer, which is worse than with other forms of cancers. Ironically, the Pap smear

test is more invasive than a simple oral exam; however, it is far more accepted by the public and has hence brought down the mortality rate of the disease significantly (Smart, 1993). This indicates that screening tests lead to earlier detection of cancer, improve prognosis and save lives.

1.7. TB as an adjunct tool in identification of malignant and premalignant lesions

One of the most challenging questions that faces clinicians in the field of oral cancer prevention is who to identify premalignant lesions that have the potential to transform into carcinoma. A possible key to improvement in diagnosis and prognosis of oral cancer may be the use of a dye solution called toluidine blue. This solution can be used to identify malignant tumors and premalignant lesions with malignant transformation potential. In addition, TB may aid in selecting biopsy sites for lesions that are clinically difficult to determine. This thesis will study the relationship between the positivity of TB, LOH and clinical prognosis in OPLs.

1.7.1. TB as a topical visual aid

Before TB became known as a facilitating tool in clinical diagnosis of oral cancer, it had another use in early twentieth century medicine. Early investigators discovered an antiheparine effect for this dye, leading to its use as a therapeutic agent in the treatment of some bleeding disorders in the late 1940s (Mashberg, 1983). After many years of usage of and research on TB, the primary use of the dye has shifted from therapeutic agent to a

topical visual aid that facilitates diagnosis of cancer. There are two methods of topical application of TB that are used in the study of oral lesions.

Rinse Method: The physician asks the patient to do the following: 1) rinse his/her mouth with 1 % acetic acid for 20 seconds; 2) rinse with water for 20 seconds; 3) rinse and gargle with 10 ml of 1% of TB for 10 seconds, twice; 4) rinse with 150 ml of 1% acetic acid for 1 minute; and 5) rinse with water. (Depending on practitioners, the pre-rinse with acetic acid before TB application may be omitted, and the time of second acetic acid rinse may vary between 1 minute to 20 seconds.)

Normally, a distinct, dark blue intense staining of a lesion is a 'positive' result. If a lesion stains only slightly, it is considered a 'weak' or 'equivocal' result. A 'negative' result shows no sign of dye pick-up in a lesion.

If positive staining is observed in any soft tissue, photographs are taken. Reevaluation usually follows after 10 – 14 days to allow any inflammation or ulcer to heal before a second rinse or direct TB application. If the lesion is still positive, it is biopsied for histological evaluation.

Direct Application Method: The procedure requires that the patient: 1) rinse his/her mouth with water for 20 seconds, twice; 2) rinse with 1% acetic acid for 20 seconds; 3) then the area is dried by the physician/ dental practitioner with gauze and; 4) a 1% TB solution is

applied with a cotton swab to the specified/ suspicious area; 5) again rinse with 1% acetic acid for 1 minute and finally; 6) rinse with water.

Positive cases are evaluated on a second occasion as previously described. If a second positive retention of the dye is seen, biopsy is recommended.

The differential stain between *CIS*/SCC and normal tissue depends on the time between dye retention and release of the dye with the rinsing procedure. Normally, cancerous tissue retains the dye for several hours, while normal tissue stains for a very short time and releases the dye after rinse with acetic acid (Bernal, 2002).

1.7.2. TB as a tool for identification of cancer

After the discovery of the antiheparine effect of TB, investigators started to explore the dye's other potential medical uses. In 1963, Richart and his colleagues discovered that the dye would stain areas of carcinoma *in situ* of the uterine cervix but not the normal epithelium (Gary *et al.*, 1986). This astonishing finding led to a new path in the research and usage of the dye.

Myers (1965) was the first scientist to test the use of TB in staining of melanoma, fibrosarcoma, lymphosarcoma, and epidermoid cancer. Some of his seventy patients also had suspicious lesions in the oral cavity. The positively stained lesions were biopsied immediately, and negatively stained cases were also biopsied to investigate their nature.

The results of his study showed that most of the negatively stained lesions were leukoplakia, hyperkeratosis, and lichen planus as well as some inflamed or ulcerated lesions. All such negatively stained lesions were confirmed to be benign upon histological exam. Some other lesions with ulcers or inflammation stained weakly. Many positively stained lesions were diagnosed as malignant melanomas (fibrosarcoma or lymphosarcoma) or epidermoid carcinomas. These lesions exhibited very dark, intense staining with one exceptional case of recurrent adenoid cystic carcinoma of buccal mucosa, which did not pick up the dye. A possible explanation for this failure to stain could be that the lesion was not ulcerated or was completely covered by mucosa, which could have blocked the stain from reaching the tumor. From these results, Myers and his research team suggested that it might be necessary to place more attention on clinical exam and the experience of the clinician rather than relying solely on TB staining for identification of carcinomas.

Despite some early questions raised about the capabilities of TB, numerous investigations in the last couple of decades have led to a better understanding of the way in which this dye can be used to assess malignancies. In 1981, Mashberg discovered that the dye identified not only visible large lesions but also very small second primary lesions in patients with malignant tumors (Mashberg, 1981). This discovery was incidental and gave TB more value and significance because those second primary lesions are usually hard to detect clinically. Since this finding, TB rinse has been suggested for inclusion as a routine procedure in a clinical exam.

1.7.3. TB as a tool for identification of oral squamous cell carcinoma

The introduction of TB usage in identifying oral SCC came soon after the discovery by Richart *et al.* of carcinoma *in situ* of the uterine cervix. Niebel and Chomet in 1964, and Shedd *et al.* in 1965, evaluated the use of TB in the identification of oral malignancies in a similar manner as seen in Richart *et al*'s study. It was found that the dye had an ability to identify multiple lesions and to differentiate tumors from normal tissues in oral cancer (Strong *et al.*, 1968). Furthermore, it was revealed that TB could identify many different types of oral malignancies. For example, the study conducted by Shedd *et al.* (1965) showed positive staining in erythroplakic carcinoma *in situ*, early small invasive carcinoma, large invasive carcinoma, leukoplakic malignant tumors, and persistent postirradiation carcinoma. As expected, dysplastic lesions exhibited less intense staining (Mashberg, 1983).

In a study by Reddy *et al.* in 1973, all of their carcinomas of the oral cavity (100%) showed positive staining. Similarly, in 1996, Warnakulasuriya and his colleagues tested the dye for the detection of oral cancer, and they found all of their carcinomas stained positive as well. The results of other studies conducted between the 1960s and the 1990s are summarized in Table 4.

As research on the efficacy of TB continued, a new idea for using this dye was tested by some researchers. In 1981, Mashberg compared the two different application methods described in section I.7.1 (direct application and rinse procedures) to see if the different

application of the dye affected the ability to recognize squamous cell carcinomas. The results showed that TB in the direct application detected 50 SCC out of 51 cases. With the rinse procedure, 48 out of 51 SCC cases were recognized as malignant tumors. The slightly higher accuracy for the direct application method suggested that it be used rather than the rinse method for facilitating clinical exam of suspicious lesions. However, as mentioned in the previous section, Mashberg discovered that 4 clinically unapparent second primary cancers (missed by direct application method also) were identified by rinse. Although TB by rinse may not yield results as accurately as the direct application, it is still an excellent tool for detecting unobserved, asymptomatic oral mucosal cancers (Mashberg, 1981).

An interesting finding was noted from Myers *et al.* study in 1965. They found that it is possible for benign and malignant lesions to coexist in the same patient at the same time. For example, one of his subjects had epidermoid cancer on the floor of the mouth and also leukoplakia (in this case, benign) in the adjacent area. In this patient, the malignant tumor had TB positive staining, but the benign lesion did not stain at all. Similarly, Sidransky and his colleagues examined 46 lesions including normal, dysplasia, *CIS,* and SCC. They discovered that some of the patients had positively stained tumors coexisting with negatively stained adjacent lesions (premalignant). Upon molecular analysis, it was revealed that in two-thirds of such cases, the same genetic changes were shared between tumors and their adjacent lesions (Sidransky *et al.*, 2001). Thus, most of the positively stained tumors and their negatively stained adjacent lesions had undergone the same genetic alterations. This suggests that one genetically altered cell expands upon gaining the growth advantage and spreads over the oral mucosa, and that the cells in the adjacent lesions have the same clonal

origin as the tumors even though the lesions have not yet developed into cancer at this stage. Therefore, positively staining adjacent lesions have a higher risk of malignant transformation compared to the negatively stained adjacent lesions that do not share the same genetic changes as the tumors.

The delineation of oral carcinomas is usually not an easy task because 1) tumors usually start out as surface lesions which spread over the surface of mucosa and sometimes merge together with surrounding inflammation or leukoplakia (Strong *et al.*, 1968); 2) some lesions are very small, asymptomatic, or morphologically similar to benign lesions and are easily missed clinically; 3) at the early stage of cancer, color changes may not be very different from the changes seen in the surrounding normal mucosa; 4) sometimes, the only clue to the detection of such lesions is the area of erosion, where the thin covering layer of keratin is missing (Strong *et al.*, 1968); and 5) due to the unique characteristic of multicentric origin of oral cancer, it is not unexpected for multiple or satellite tumors to exist adjacent to the main tumor mass. They are known to be missed easily by clinical exam (Strong *et al.*, 1968). To identify these lesions, the dye has an additional role to play in selecting ulcers that need biopsy. Such ulcers are suspected of being cancerous and are very difficult to differentiate from benign ulcers. The suspicious lesions tend to have diffuse stain on the margin, while benign ulcers have distinct, well-defined stain on the edges (Bernal, 2002).

In summary, with appropriate techniques and in the hands of experienced personnel, TB has potential value in the following situations: 1) differentiation of hyperplasia or dysplasia from carcinoma; 2) differentiation of ulcers or inflamed lesions from carcinoma; 3) detection of

second, third, or more primary lesions; 4) identification of clinically undetected lesions; and 5) determination of site and margin of biopsy (Myers *et al.*, 1965).

Table 4. Summary of earlier studies with TB in identification of SCC[1]

Study	Year	# of SCC cases examined[2]	# of carcinomas detected by TB[3]
Niebel and Chomet	1964	11	11
Shedd *et al.*	1965	44	44
Shedd *et al.*	1967	62	62
Myers	1970	71	69
Rosen *et al.*	1971	45	26
Vahidy *et al.*	1972	1030	886
Reddy *et al.*	1973	490	485
Sigurdson and Willen	1975	54	54
Silverman *et al.*	1984	132	129
Epstein and Scully	1992	59	55
Barellier *et al.*	1993	235	225

[1]Adapted from Johnson, 1998.
[2]The total number of squamous cell carcinomas confirmed upon histological exam.
[3]The number of carcinomas detected by positive staining of TB.

1.7.4. TB as a tool for identifying OPLs

Four decades of research on TB have lead to the recognition of the use of this dye as an effective tool in detecting oral malignancies. However, its ability to differentiate premalignant lesions with cancer transformation potential from other premalignant lesions without such potential is still being questioned. Along with malignant lesions of oral cancer,

some premalignant lesions have been tested with TB. Despite much effort and research, all the results from such studies are inconclusive. Some have reported positive staining results for all of their premalignant lesions and others have reported mixed results (some lesions with positive staining and some with negative). For instance, Silverman *et al.* reported the TB staining test to be 100% accurate for dysplasia in their 1984 experiment. In that study, all of the histopathologically confirmed 42 epithelial dysplasias displayed dark blue positive staining. Because 100% sensitivity of TB is very rarely seen with premalignant lesions, some investigators doubt Silverman's results, stating that their study examined only patients with obvious lesions and compared biopsy specimens to the presence or absence of staining (Martin *et al.*, 1998). In other words, their study was not totally blinded, thus there was some bias generated in the results. They might have neglected those dysplastic lesions that were clinically normal and did not look to be 'obvious' abnormal lesions. Furthermore, Silverman *et al.* did not investigate the malignant transformation potential of dysplastic lesions. Therefore, '100% sensitivity' does not imply that all of those positively stained precancerous lesions will progress into carcinomas.

Other studies also contribute to a consensus that TB is inconsistent in staining premalignant lesions. When Gary and his colleagues (1986) examined 75 patients with clinically normal oral tissues, they observed positive staining in 16 patients with TB rinse method and 6 patients with the direct application method. The histological examination of these biopsy specimens revealed that there was no evidence of dysplasia or carcinoma; however, "benign tissue abnormalities" such as hyperkeratosis were seen in some of the lesions. Again, they did not investigate the malignant transformation potential of lesions that stained positive.

Also, no conclusion can be drawn about what causes some precancerous lesions to stain and not others. The results of other investigations of TB staining in premalignant lesions are tabulated in Table 5.

There is one limitation when reading the results from various researchers. Depending on the investigators, the definition of negative staining may vary. For instance, one may record a weakly stained lesion as negative whereas others may call it equivocal or even positive. Since there is no standardization of what is positive, negative and equivocal staining, this may cause a great deal of confusion and misinterpretation of results.

Table 5. Summary of results of TB staining of OPLs

Author	Year	Histological Diagnosis	Total # of cases	TB +	TB -
Reddy *et al.*	1973	Mild dysplasia	33	31	2
Silverman	1994	Dysplasia[1]	13	11	2
Warnakulasuriya	1996	Dysplasia[1]	13	5	8
Martin *et al.*	1998	Mild/ moderate dysplasia	40	17	23

[1]The degree of dysplasia was not reported.

1.7.5. TB staining of other oral lesions

Sometimes benign ulcers and inflammatory lesions, which possess no risk of malignant transformation, are reported to be TB positive. This is because inflammation and ulceration

are believed to cause erosion on the surface of the lesion, increasing the ability of the lesion to pick up and retain the dye. However, this staining mechanism is not fully understood. It is important to note that much of this false positive staining is eliminated by the inclusion of a repeat stain to positive lesions after a healing period of 10-14 days. The majority of commercial products now recommend a waiting period between two examinations with the dye to allow transient inflammatory conditions to heal (Johnson, 1998).

1.7.6. Sensitivity and specificity of TB in detection of SCC

Several publications have reported sensitivity and specificity values for the ability of TB to detect SCC. Sensitivity/true positive rate (sometimes referred to as false negative) represents how sensitive the dye is to carcinomas. It is used to establish the frequency at which negative staining is seen for SCCs. The sensitivity of TB is almost always very high, ranging from 90% to 100%. A few rare reports exist of a low sensitivity (Rosen *et al.*, 1971; Warnakulasuriya, 1996). It has been suggested that in such cases, the failure to stain in the presence of SCC could be caused by the smaller amount of TB solution used in a procedure when compared to other studies, so the dye might not have been able to reach all sites (Warnakulasuriya, 1996).

The specificity/true negative rate (sometimes referred to as false positive) indicates TB positive staining rates for lesions that are not carcinomas. The lowest specificity ever recorded was approximately 50%, but it usually ranges from 63% to 94% with a single rinse of TB (Rosen *et al.*, 1971; Epstein and Scully, 1992; Silverman, 1994). These figures can be

raised much higher to a range of 88% to 99% with a second rinse after the waiting period of 10 to 14 days, which allows for traumatic ulcers and inflammatory lesions to heal (Warnakulasuriya, 1996).

The specificity is also greatly affected by the experience of the clinicians and nurses who perform the procedures. This is clearly illustrated in a big study conducted in 1973 by Reddy *et al.* As many as 9,400 patients were assessed by inexperienced staff, and the false positive and negative rates were very high. In comparison, when more experienced staff reassessed the patients, the numbers dropped significantly (Reddy *et al.*, 1973). From this, researchers learned that even this easy-to-perform TB application requires the expertise of clinicians in order to produce accurate results.

In the investigation of sensitivity and specificity of TB, numerous researchers have conducted studies to confirm its usefulness in the medical field. For example, Warnakulasuriya and his colleagues examined TB's reliability to differentiate carcinomas and dysplasia from benign keratotic lesions. Their result showed all invasive carcinomas stained positive, giving TB the sensitivity of 100%. 11 out of 29 benign keratosis, hyperplasias, and ulcers stained positive, giving the specificity of 62% (Warnakulasuriya, 1996). This may suggest that TB can be used as a screening tool for a high-risk population, but not for the general public because the high false positive rate may create some confusion in the results. However, it is difficult to draw a strong conclusion when a study includes few subjects, as in Warnakulasuriya's study. In addition, the researchers used only one rinse in

their procedure and did not perform a second rinse with a proper waiting period in between, which might have decreased the false positive rate dramatically.

Mashberg (1981) also investigated the sensitivity of TB and compared the results of application and rinse methods in oral cancer, as mentioned earlier. That result showed 98% sensitivity (false negative of 2%) with the direct application and 94% with the rinse method (false negative of 6%). This lower sensitivity of the rinse can be partially explained by less intense stain in the rinse technique compared to the direct application method. Positive results of TB staining were also seen in nonmalignant lesions (5 with the direct application and 4 with the rinse) of 54 nonmalignant lesions. This implies that the application and rinse have specificities of 91% and 93% respectively. Like Warnakulasuriya, Mashberg also suggests the dye be used for screening high-risk populations. The sensitivity and specificity of other studies are tabulated in Table 6.

In 1996, another research team assessed the efficacy of TB as a general public screening method. Epstein *et al.* estimated that if TB were used as a screening test in the general population, there would be only 6% of PTL+ (Post Test Likelihood of +ve test, which is the probability that the disease is actually present). However, PTL+ would be increased to 51% if used in patients who have had upper aerodigestive cancer (Epstein *et al.*, 1996). Therefore, he agrees with other researchers that TB might be better used for screening a high-risk population rather than the general public.

Table 6. Review of sensitivity and specificity of TB in detection of SCC[1]

Author	Year	Sensitivity (%)	Specificity (%)
Niebel and Chornet	1964	100%	[2]NR
Shedd *et al*	1965	100	85
Myers	1970	98	100
Rosen *et al*	1971	50	50
Reddy *et al*	1973	99	83
Lundgren	1979	91	51
Mashberg	1981	98 (direct application) 94 (rinse)	91 (direct application) 93 (rinse)
Silverman	1984	98	70
Epstein and Scully	1992	93	63
Silverman	1994	94	94
Warnakulasuriya	1996	100	62
Epstein and Scully	1997	100	52

[1]Adapted from Johnson, 1998.
[2] NR=not reported in the study.

1.7.7. Sensitivity and specificity of TB stain in detection of dysplastic lesions

In comparison with SCC, there has been far less research done on the efficiency of TB in detection of premalignant lesions, especially those with malignant transformation potential. The investigations with early precancerous lesions in the past have shown inconsistent staining results, especially with mild dysplasia. Unlike malignant lesions, which normally have intense solid staining characteristics, premalignant lesions can have solid, speckled or faint discoloration patterns as well as no staining (Warnakulasuriya, 1996).

Since no well-established evaluation criteria exist for premalignant staining, variation in interpretation of results may appear. For instance, some may call a weak staining of premalignant lesions, which is often observed, equivocal. Others may call it positive or even negative. If a researcher calls it positive, it will create greater false positive ratings for his/her study than others who call it equivocal or negative. Moreover, if a study categorized dysplasias together with malignant lesions, their findings could be misinterpreted by other researchers who separate dysplastic lesions from malignant ones in their staining results. For example, one study may interpret dysplasias and malignant lesions together in one group as TB positive or negative; another study may place results with dysplasia separate from malignant lesions. In the former case, a higher false positive rate is expected because even less intensive staining patterns of some dysplastic lesions, otherwise considered equivocal, are considered as positive along with the positive staining of malignant ones.

The reason why some researchers and clinicians treat dysplasias together with malignant lesions is that dysplasias have a higher risk of transformation into malignancy than any other premalignant form such as hyperplasia or hyperkeratosis. Therefore, they often think that it would be better to have a higher false positive rate than to have a higher false negative rate and run a risk of under diagnosis, which in turn leads to the development of more advanced forms of lesions.

As demonstrated in the examples above, the variation among different study groups, the perplexing staining coding for dysplasia, and the inconsistency in staining results may have been discouraging factors in assessment of sensitivity and specificity of TB in premalignant lesions in the past. However, despite the small number of studies done on this subject, there seems to be a trend created in the results.

Premalignant or dysplastic lesions are expected to have much lower sensitivity rates than SCCs. For example, in the same study mentioned earlier, Warnakulasuriya (1996) showed that 29 out of 39 dysplastic lesions stained positive, eight stained negative and two had equivocal staining. This represents a sensitivity of 78 % for identification of dysplasias, much lower than the sensitivity of 100% observed in detection of SCC. Other studies also add to this trend: a 42% sensitivity for mild-to-moderate dysplasia was reported in 1998 by Martin *et al*, and a 75% sensitivity for dysplasia was reported by Mashberg in 1980. It is interesting to note that there has been a rare case of high sensitivity reported in the past. Silverman (1984) reported a 100% sensitivity for TB assessment of dysplasia. However, a

number of investigators have been reluctant to accept Silverman's result due to the reasons already discussed earlier.

Unlike the study of Warnakulasuriya (1996), Gary and his fellow researchers (1986) added a second rinse 14 days after the first test in their study in an attempt to obtain more reliable sensitivity in detection of early neoplastic changes in oral cavity, which appeared clinically normal. This waiting period allowed most ulcers and inflammatory areas to heal before the second test. Their results, as described earlier in section I.7.4, showed that 16 out of 75 patients without any morphological changes stained positive with rinse. 6 such patients stained positive with the direct application (Gary *et al.*, 1986). Since no dysplasia or carcinoma were confirmed in any of the cases, it would give both rinse and application zero sensitivity and a specificity of 79% and 92% respectively. This finding, along with the other investigations mentioned in I.7.6 section, suggests that TB may not be best used for the detection of early lesions of clinically normal patients and not suitable for screening the general public. The sensitivity and specificity rates of premalignant lesions are summarized in Table 7.

Although TB seems to create inconsistent staining with early premalignant lesions, it is still a great aid for identification of carcinomas and high-risk premalignant lesions when used in conjunction with clinical exam. Having a well-established evaluation criteria/coding for premalignant lesions and understanding the mechanisms of how and why certain premalignant lesions stain positively with TB would certainly improve our ability to use this dye in screening high-risk lesions.

Table 7. Summary of sensitivity and specificity for dysplasias

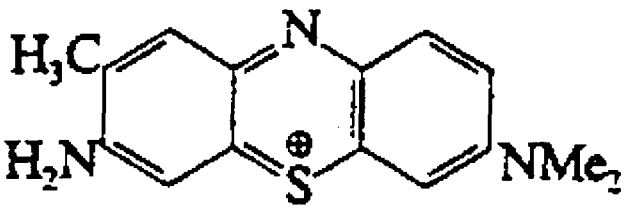
Author	Year	Sensitivity (%)	Specificity (%)
Reddy *et al.*	1972	87	89
Gary *et al.*	1986	79	92
Silverman *et al.*	1994	85	94
Warnakulasuriya	1996	78	62
Johnson[1]	1998	~50	~89
Martin *et al.*	1998	58	NR[2]

[1] rough estimates from previous studies
[2] NR= not reported in the study

1.7.8. Mechanism of staining

Toluidine blue is a metachromatic thiamine dye with molecular weight of 305. It is soluble in both water and alcohol (Strong *et al.*, 1968). The dye has an affinity for acidic components of tissues, thus it binds with DNA and RNA. This acidophilicity comes from the thiazine group that selectively attracts and stains acidic tissue components such as sulphates, phosphates (especially, the phophate group of nucleic acids), and carboxylates (Martin *et al.*, 1998). Since these acidic components can be observed in an increased quantity in cells that undergo neoplastic proliferation, the positive staining of this dye would result (Dunipace *et al.*, 1992).

Figure 3. Chemical structure of TB



(Adopted from Dictionary of Organic Compounds, 1996)

When cells are subject to abnormal, uncontrolled proliferation, the rate of DNA synthesis is increased as well as its products. Therefore, dysplastic cells have an increased amount of nucleic acids, compared to normal cells. Cancer cells have even higher concentrations of nucleic acids than dysplastic cells. This is believed to be the strongest cause of the positivity of TB staining seen in such lesions.

The healthy oral epithelium has a very effective barrier system with selective permeability. The normal epithelium surface is known to be lipophilic, and TB is a hydrophilic solution. Therefore, the dye is usually prevented from penetrating. However, in oral cancer, it is suspected that the top keratin layer of carcinomas may be damaged through the carcinogenesis process and its barrier system may become defective, allowing for the uptake of the dye (Epstein, 1992). Furthermore, malignant cells are known to have wider intercellular canals than normal cells, which allow for more penetration of the dye. Also, sometimes, carcinomas and even some dysplasias have looser cell arrangements, thus increasing the uptake of the dye and allowing the deposition in intercellular spaces (Epstein, 1992).

The widening of intercellular canals is also a common phenomenon in ulceration. This feature along with erosion of the surface of an ulcerated lesion is considered to be the main cause for the uptake of the dye in inflamed or ulcerated tissues. TB solution penetrates these widened intercellular canals and is absorbed by the nucleated cells that are located underneath and now exposed to the dye due to the erosion.

Unlike ulcers, hyperkeratosis does not stain, due to the presence of keratin on top which prevents the entry of the dye. Also, leukoplakia does not usually pick up the dye because it has no nuclei in the surface layer of keratin and it appears to have no intercellular canals (Strong *et al.*, 1968). Parakeratosis, however, does stain because its superficial layers contain nuclei (Reddy *et al.*, 1973).

While some authors believe that the deep staining of TB in carcinomas is strictly due to the increased amount of nuclear materials, others argue that it is actually caused by the diffusion of the dye through several superficial cell layers in which the cells are arranged haphazardly or more loosely (Strong *et al.*, 1968). For example, in the study of Strong and his colleagues, it was shown that the stain was not picked up primarily by the nuclei but diffused between the tumor cells to a depth of three to four cell layers in thin frozen sections of tumor. Despite such arguments, both of these characteristics (the high density of nucleic acids and loose cell-arrangement) are usually observed in most malignant cells. It can be speculated that it is the combination of these two factors plus the additional feature of widened intercellular canals that causes the most intense staining in these malignant lesions.

Unlike all investigations mentioned above, one interesting report documented that the staining of SCC is mainly caused by the mitochondria of living carcinoma cells (Bernal, 2002). Bernal claims that what attracts TB in malignant cells is the negative charge that resides inside mitochondrial membrane. In cancer cells, there seems to be a higher mitochondrial electrical potential than normal cells. Therefore, this stronger charge attracts more dye, allowing cancer tissues to stain dark blue (Bernal, 2002). Mitochondria of normal cells do take up the dye but release it quickly after rinse with dye-free solution. While the dye is normally not very visible to human eyes in normal cells, it is retained for about 80 minutes in living carcinoma cells (Bernal, 2002). He emphasizes that this phenomenon occurs only if cells are alive. If cells are dead or fixed on slides, the dye appears to concentrate in nuclei, not in mitochondria. Since this is the only report, to my knowledge, investigating mitochondria and its uptake of TB solution, the repetition of such a study is necessary.

As introduced earlier in this thesis, TB's most recognized value comes from its ability to differentiate carcinomas from normal tissues by staining deep blue and from its high sensitivity for carcinomas. However, there are some documents reporting its limitations.

For example, it has been reported that the dye cannot stain the deep, submucosal extension of the tumor as sometimes occurs in some patients because TB cannot penetrate through the keratin layer or deep into submucosally located lesions unless eroded (Reddy *et al.*, 1973; Strong *et al.*, 1968). Furthermore, not only is the dye taken up by the malignant lesions, it is

also picked up by food debris, bacteria, and purulent exudates (Strong *et al.*, 1968). In addition, the accumulation of the dye can be seen in small fissures or the pores of seromucous glands as well as the nucleated scales covering the papillae on the dorsal surface of the tongue. However, the latter does not usually cause any confusion in the interpretation of the TB result. Again, the experience of the clinicians and precision of the procedure makes a considerable difference in producing accurate results and success of TB use in cancer detection.

1.7.9. Molecular basis for TB staining in dysplasia

In premalignant lesions such as dysplasia, the reports on TB staining patterns have been very inconsistent. In my own review of the literature on this issue, the positive staining of dysplasia was found to vary from a low of 33% (Reddy *et al.*, 1973) to a high of 87% (Mashberg *et al.*, 1981). The reason for this inconsistency in results is not known.

The trend seen in the staining of dysplasia is TB positive in severe dysplasia (and *CIS*) and negative in lower grade dysplasia. The frequent positive results of *CIS* and severe dysplasia can be explained by the fact that these lesions are morphologically similar to SCC and possess all the defective characteristics of carcinomas mentioned in the previous section. A question arises with mild dysplasia, which is famous for its inconsistent staining. What causes this difference in staining? One possible answer to this question is that mild dysplastic lesions that stain with TB may also have all or some of the defective features seen in carcinomas, such as increased amount of nuclei, wider intercellular canals, loosely

arranged cells, and defective barrier in the superficial layer. The possible cause for these defective characteristics in the staining lesions may be hidden in the genetic alterations. This is to say that the positive mild dysplasia may contain similar genetic changes to those observed in malignant lesions. Such changes, in turn, may create the defective phenotypes. On the other hand, non-staining dysplasias may have fewer of these genetic changes and thus very little or none of the phenotype that causes the uptake of the dye. In this thesis, microsatellite analysis of LOH will be used to investigate the possible molecular differences seen in these lesions.

2. STATEMENT OF PROBLEM

Only a small percentage of OPLs without dysplasia or with low-grade (mild/moderate) dysplasia will progress into cancer. Identification of progressing high-risk lesions is of vital importance. A recent study from this laboratory has shown that different profiles of loss of heterozygosity (LOH) in biopsies of OPLs are associated with low (retention at 3p and 9p, RR = 1), intermediate (LOH at 3p &/or 9p, RR = 3.75) or high cancer progression risk (LOH at 3p &/or 9p plus loss at any of 4q, 8p, 11q, 13q or 17p, RR = 33.4) (Rosin *et al.*, 2000). Molecular markers, however, will be valueless if high-risk oral premalignant lesions are not biopsied, or are biopsied, but not from the highest risk areas of oral premalignant lesions. The determination of when to biopsy a lesion and where to locate the biopsy is a continuing challenge facing clinicians. Currently many oral specialists use toluidine staining to help such determination and the literature suggests that in experienced hands TB staining facilitates the identification of dysplastic oral premalignant lesions. There is little information on whether dysplasia identified by positive TB staining have a different molecular pattern and cancer risk from those with similar degree of dysplasia but negative for TB staining. For example, the phenotype of TB positive staining could reflect increased nuclear content at the superficial layers of the epithelium and/or a defective intercellular barrier, both of which could be controlled by genetic changes. Do toluidine positive dysplasias differ genetically from toluidine negative dysplasias?

3. OBJECTIVES

1) To determine the TB staining results in OPLs that are currently being followed up in a large longitudinal study.

2) To characterize the pattern of genetic changes in the above oral premalignant lesions by means of LOH analysis using 11 microsatellite markers for the 3 chromosomal regions known to be frequently lost in oral tumors: 3p14.2 (*D3S1234, D3S1228, D3S1300*); 9p21 (*IFNA, D9S171, D9S736, D9S1748, D9S1751*); 17p11.2 (*CHRNB1*) and 17p13.1 (*tp53* and *D17S786*).

3) To compare the LOH pattern of TB positive oral premalignant lesions with those of TB negative cases.

4. HYPOTHESIS

TB positive oral premalignant lesions will show increased high-risk LOH molecular patterns and increased cancer progression as compared to TB negative cases.

If data support this hypothesis and found TB positive oral premalignant lesions with more high-risk LOH patterns, it would suggest that TB positive oral premalignant lesions have higher cancer risk than the TB negative oral premalignant lesions, and suggest that the increased changes in the critical control genes might underline the increased uptake of TB dye, and would also suggest that TB staining would not only be useful in identifying SCC and dysplasia, but also useful in identifying high-risk dysplasia or oral premalignant lesions.

5. MATERIALS AND METHODS

5.1. Sample collection

This study examined OPLs with a main emphasis on low-grade oral dysplasias. Two types of samples were used: retrospective archival paraffin-embedded samples collected from Vancouver Hospital and Health Sciences Centre and OPLs from an ongoing longitudinal study on oral leukoplakia.

The criteria for selecting cases included the following:

1) Histological confirmation by two pathologists (Dr. L. Zhang and Dr. R. Priddy, the Provincial Oral Biopsy Service, the University of British Columbia) using World Health Organization (WHO) diagnostic criteria for dysplasia, which include: loss of basal cell polarity, more than 1 layer of basaloid cells, increased nuclear to cytoplasmic ratio, drop-shaped rete ridges, irregular stratification, increased and/or abnormal numbers of mitosis in the basal compartment as well as increased mitotic figures in the superficial half of the epithelium, cellular pleomorphism, nuclear hyperchromatism, enlarged nucleoli, reduction of cellular cohesion, and keratinization of single cells or cell groups in the spinous cell layer (WHO, 1978). Dysplasia is farther divided into mild, moderate, and severe. This classification is based upon the degree of the spread of dysplastic changes throughout the epithelial layer. In mild dysplasia, such changes are restricted to 1/3 of the layer; in moderate dysplasia, the changes are seen in 2/3; and in severe dysplasia, those changes spread

to from 2/3 to the entire epithelial layer. Such severity in dysplastic spread can also be seen in *CIS*, where the whole epithelium is covered by abnormal cells but the basement membrane is still intact. When the cancer reaches the most aggressive stage of SCC, the basement membrane is broken through, and the underlying connective tissue is invaded by the cancerous cells. This is a strong indication of malignancy.

2) Confirmation that there is enough tissue to provide adequate DNA from both connective and epithelium tissues. The connective tissue serves as a source of normal DNA.

3) A record of results of TB analysis on hospital charts.

5.2. Sample sets

Group 1: 40 cases of TB positive OPLs.

Group 2: 27 cases of TB negative OPLs.

Both sets of samples included hyperplasia (without dysplasia) and samples with dysplasia.

5.3. Patient information

In addition to TB staining results and histological diagnosis of the lesions, the following patient information was collected: age, gender, site of the lesion, history of cancer, smoking habits, and outcome of the lesion, i.e., whether the lesion progressed into cancer.

5.4. Slide Preparation

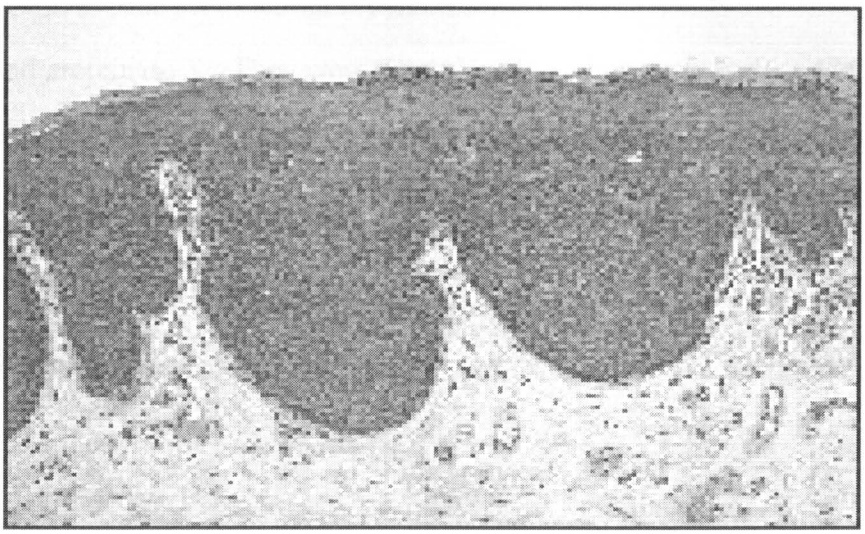
Following confirmation of diagnosis and sufficient tissue on blocks, a 5 micron section was cut from each block and stained with hematoxylin and eosin (H&E) for use as a dissection reference slide. A further 10-15 sections were cut at a 12 micron thickness for dissection. These slides were also stained with H&E. The H&E procedure is described below:
The sections were baked overnight at 37°C in an oven, then at 60-65°C for 1 hour, and were left at room temperature to cool. Samples were deparaffinized by two changes of xylene for 15 minutes each followed by dehydration in graded ethanol (100%, 95% and 70%), and hydrated by rinsing in tap water. Slides were then placed in Gill's Hematoxylin for 5 minutes, followed by rinsing in tap water, and were then blued with 1.5% (w/v) sodium bicarbonate. After rinsing in water, slides were lightly counterstained with eosin, dehydrated, and cleared for coverslipping. Thick sections to be dissected were stained by the above procedure without the dehydration step, and air dried (Michelsen, 1997).

5.5. Microdissection

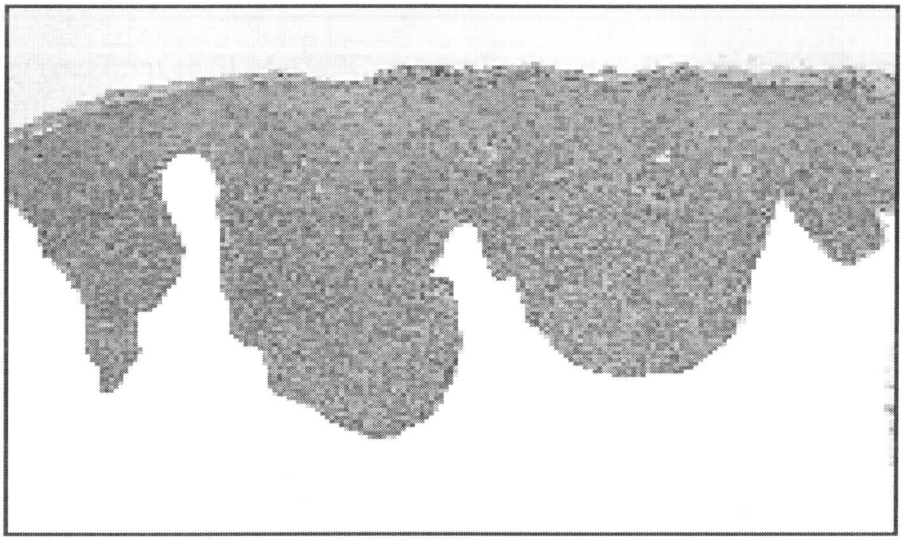
Under a dissection microscope, the epithelial layer at the basal cell membrane was separated from underlying connective tissue with a 1 ml syringe needle. The collected connective

tissue was used as a control for the study, as the epithelium tissue was used for experiment. The dissected tissues were histologically validated by the pathologist (Dr Zhang) (Figure 4). The tissues were put in a 1.5 ml eppendorf tube separately. Dysplastic cells were microdissected away from normal epithelium and collected into a separate eppendorf tube.

Figure 4. Histological view of dissection



Section before dissection.



Section after dissection. The Connective tissue layer is removed.
Epithelial part underlying the sample is collected separately.

5.6. Sample Digestion and DNA Extraction

The samples were digested in 300 µl of 50 mM Tris-HCL containing 1% sodium dodecyl sulfate (SDS) and proteinase K. They were then placed in a hot water bath at 48°C for a minimum of 72 hours. During the incubation, they were spiked with 10-20 µl of fresh proteinase K (20 mg/ml) twice a day. DNA was then extracted twice with PC-9 (Phenol-Chloroform), precipitated with 70% ethanol with glycogen and washed again with 70% ethanol. Finally, DNA samples were resuspended in LOTE (low ionic strength Tris buffer). All samples were coded prior to dissection.

5.7. DNA Quantification

Sample DNA was quantified with fluorescence analysis using Picogreen kit. The absorbance was read from an SLM 4899C spectrofluorometer. The amounts of sample DNA were determined from standard curves.

5.8. Primer-Extension Preamplification (PEP)

If DNA concentration was too small (total <100 ng), a preliminary amplification step was done prior to microsatellite analysis using primer-extension preamplification (PEP). PEP increases DNA quantities by amplifying multiple site of genome using random primers. The amplification was done in 60 µl reaction volumes that consisted of 20 ng DNA sample, 900 mM of Tris-HCL, 2 mM of dNTP, 400 µM of random 15-mers (Operon Techs: CA, USA), and 1µl of Taq DNA polymerase (Gibco, BRL: Ontario). Two drops of mineral oil were added on top to avoid evaporation. The amplification protocol included 1 cycle of pre-heat at 95°C for 2 min followed by 50 cycles of denaturation at 92 °C for 1 min, annealing at 37 °C for 2 min, and polymerization at 55 °C for 4 min. Amplification was done with an automated thermal cycler (Omigene: Steinfurt, Germany).

The primers were purchased from Research Genetics (Huntsville, AL) and included the following: for LOH on 3p (*D3S1234, D3S1228* and *D3S1300*), on 9p (*IFNA, D9S171, D9S736, D9S1748* and *D9S1751*) and on 17p (*CHRNB1, tp53* and *D17S786*).

5.9. End-Labeling

The reaction was performed in a 50 µl mixture containing the following: 38 µl of PCR-quality distilled water, 5 µl of a 10 × buffer for T4 polynucleotide kinase (New England BioLabs: Ontario), 1 µl of 10 × Bovine Serum Albumin, 1 µl of one of the primer pairs, 3 µl of T4 polynucleotide kinase (New England BioLabs: Ontario), and 2 µl of [γ-^{32}P] ATP (20

μCi, Amersham: NJ, USA). The labeling was done in a single reaction in the thermocycler at 37°C for 60 min (Michelsen 1997).

5.10. PCR Amplification for microsatellite analysis

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

The PCR products were then diluted 1:2 in loading buffer, separated on a 7% urea-formamide-polyacrylamide gels, and visualized by autoradiography. The films were then coded and scored for LOH (Zhang et al, 1997).

5.11. Scoring of LOH

PCR products migrate electrophoretically on gel depending on the size of alleles. Films were coded and scored for relative intensity of autoradiographic bands. The following is the summary of scoring:

1) Informative case: yield two bands for each allele (one maternal, other paternal).

2) Allelic imbalance: loss or marked reduction of one of the allelic bands by 50%. Recorded as LOH. These samples were subjected to repeat confirmatory analysis with an independent PCR reaction.

3) Non-informative: both paternal and maternal alleles are same size.

5.12. Statistical analysis

Differences and associations between different study groups (toluidine positive vs. toluidine negative; hyperplasia vs. dysplasia; with cancer history vs. without cancer history) were examined using either Fisher's exact test for categorical variables (gender, smoking habit, histology and LOH) or t-test for continuous variables (age). All tests were two sided. $P < 0.05$ was considered to be statistically significant.

6. RESULTS

6.1. Patient clinicopathological features and demographics

Individual patient and sample data have been placed for reference into Appendix 1. Table 8 summarizes the demographic data for all patients in this study. Patient ages ranged from 34 to 93 years, with a mean of 63. Thirty-nine percent were male and 60% had a smoking habit (ever-smoker). The majority (75%) of biopsies were taken from the ventrolateral tongue and floor of mouth, which are sites at high-risk for cancer progression (section 1.4.3.).

TB positive and negative cases were compared for the aforementioned clinicopathological features. No significant association was observed between TB uptake and gender, site distribution or smoking history (Table 8, all have $P > 0.05$). However, TB positive lesions were more frequently observed in older patients (mean age 67 compared to 57 for cases with TB negative lesions ($P = 0.03$).

Table 8. Demographic features of patients

	All cases	TB positive cases (%)	TB negative cases (%)	P value[1]
Total	67	40 (60%)	27 (40%)	
Mean age (yr) ± SD	63 ± 15	67 ± 15	57 ± 13	**0.03**
Male sex -- no. (%)	26 (39%)	18/26 (69%)	8/26 (31%)	0.31
Tobacco use ever -- no. (%)	40 (60%)	23/40 (56%)	17/40 (43%)	0.80
Cases located on the ventrolateral tongue and floor of mouth (%)	33 (49%)	23/33 (70%)	10/33 (30%)	0.21

[1]TB positive vs. TB negative cases

6.2. TB staining and histology

The majority of the 67 oral premalignant lesions in this study were either hyperplasia (25 cases, 37%) or low-grade (mild/moderate) dysplasia (32 cases, 48%), with 10 cases of severe dysplasia (15%). Of the 67 lesions, 40 stained positive for TB and 27 were negative.

Table 9 examined the association between toluidine staining and the histology of the lesion (presence and degree of dysplasia). Figure 5 shows examples of various TB staining results. When the hyperplasias were compared to all dysplasias, no significant difference was seen although a higher proportion of dysplastic lesions stained positive for TB (67%) compared to the non-dysplastic lesions (48%) (P = 0.19). Similarly, there was no difference in TB uptake between hyperplasia (48% positive) and low-grade dysplasia (59%, $P = 0.43$). However,

high-grade dysplasia showed a significantly increased uptake of TB (90%) compared to non-dysplastic lesions ($P = 0.03$) or compared to hyperplasia and low-grade dysplasia ($P = 0.04$).

Table 9 also compared TB staining results for the non-dysplastic lesions (hyperplasias) with all dysplastic lesions to determine the sensitivity and specificity of TB as a tool for identifying dysplasia. Twenty eight of the 42 dysplasias demonstrated an uptake of the dye, yielding a 67% sensitivity rate. Hence the false negative rate for toluidine staining in this study is 33%. Thirteen of twenty five non-dysplastic lesions were negative for toluidine staining, indicating 52% specificity. Thus the false positive rate is 48%.

Table 9. Sensitivity and specificity of TB stain

TB result	Hyperplasia	Dysplasia	P value
# of cases	25	42	
Positive	12/25 (48%)	28/42 (67%)[1]	0.19
Negative	13/25 (52%)[2]	14/42 (33%)	

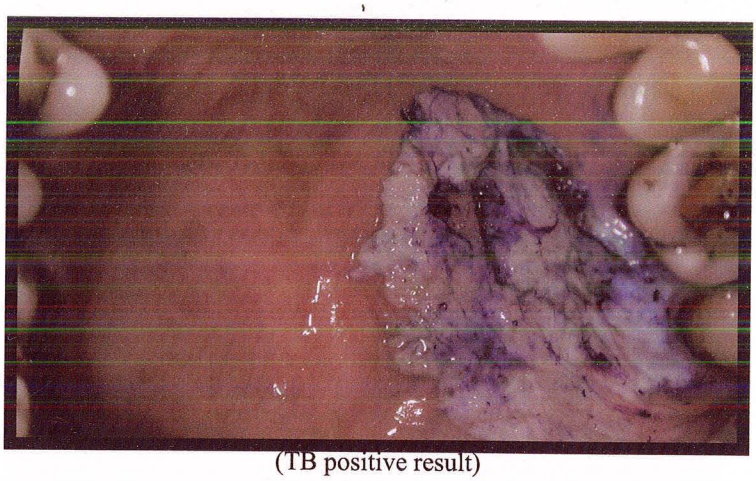
[1]Sensitivity rate. [2]Specificity rate.

Table 10. Toluidine staining in hyperplasia, low- and high-grade dysplasia

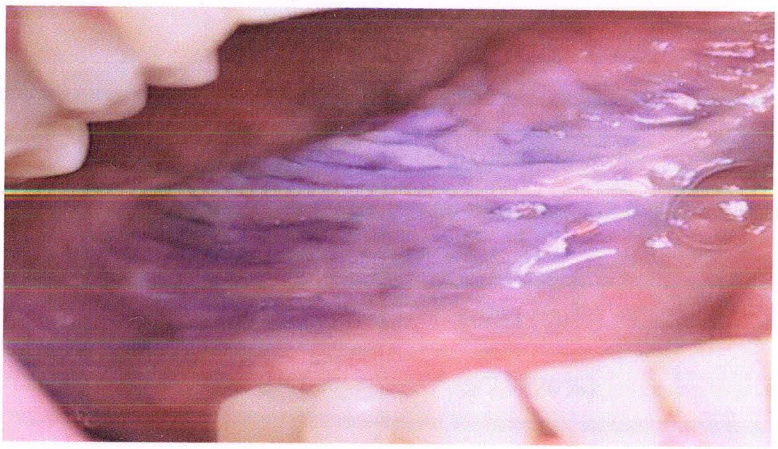
	All	Hyperplasia	Low-grade dysplasia	*P value*[1]	High-grade dysplasia	P value[2]
# of lesions	67	25	32	-	10	
TB positive	40	12/25 (48%)	19/32 (59%)	*0.43*	9/10 (90%)	**0.03**
TB negative	27	13/25 (52%)	13/32 (41%)		1/10 (10%)	

[1]Comparison between hyperplasia and low-grade dysplasia. [2]Comparison between hyperplasia and high-grade dysplasia.

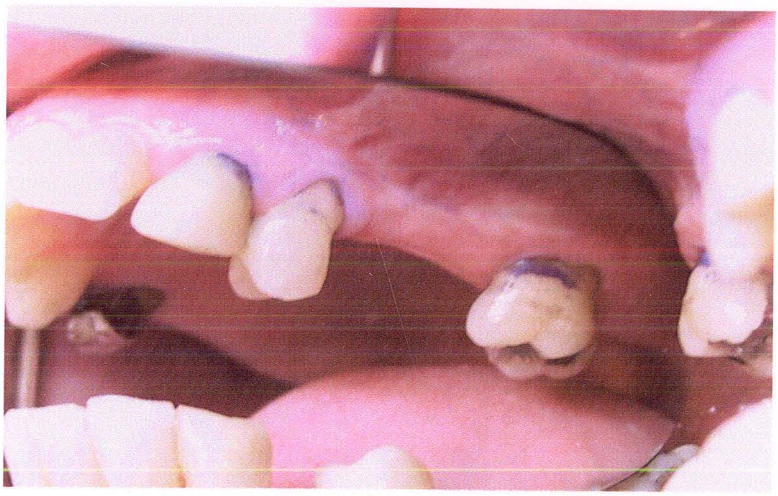
Figure 5. TB staining results



(TB positive result)



(TB weak positive)



(TB negative result)

6.3. TB staining and cancer history

Data were analyzed for a possible relationship of TB staining to history of head and neck cancer (Table 11). Of the 67 lesions, 25 were from patients with a history of head and neck cancer. These lesions showed increased uptake of the dye. Twenty of 25 (80%) lesions from patients with history were TB positive compared to 20 of 42 (48%) lesions from patients without the history ($P = 0.01$).

Table 11. TB results in patients with or without cancer history

	Without a history of oral cancer	With a history of oral cancer	P value
# of lesions	42	25	
Toluidine positive	20/42 (48%)	20/25 (80%)	**0.01**
Toluidine negative	22/42 (52%)	5/25 (20%)	

6.4. LOH and histology

Figure 6 shows an example of LOH. Table 12 compares LOH frequencies in non-dysplastic lesions (hyperplasia) and dysplasias. Increased frequencies were observed for dysplasias. These increases were marginally significant for more than 1 arm lost (40% vs. 16% in hyperplasia, $P = 0.06$), and significant for more than two arms lost (17% vs. 0%, $P = 0.04$), for 17p loss (43% vs. 8%, $P = 0.003$) and for 3p and/or 9p plus 17p lost (36% vs. 8%, $P = 0.02$).

When the dysplasias were further divided into low- and high-grade dysplasias, the data shows that the differences between dysplasia and hyperplasia were derived mainly from the high frequency of allelic loss that characterized the high-grade dysplasia (Table 13). When hyperplasia and low-grade dysplasia are compared, an increased LOH is observed only at 17p (31% vs. 8% in hyperplasia, $P = 0.05$). In contrast, high-grade dysplasia showed elevated frequencies for > 1 arm lost (90% vs. 16% in hyperplasia, $P < 0.0001$), for > 2 arm lost (60% vs. 0%, $P = 0.0001$), at 3p (70% vs. 24%, $P = 0.02$), at 9p (90% vs. 40%, $P = 0.01$), at 17p (80% vs. 8%, $P < 0.0001$) and for 3p and/or 9p plus 17p (80% vs. 8%, $P < 0.0001$, Table 13) when compared to hyperplasia. Similarly, high-grade dysplasia showed significantly increased frequencies of LOH compared to the low-grade dysplasia for all categories (Table 13).

Table 12. LOH frequencies in hyperplasia and dysplasia

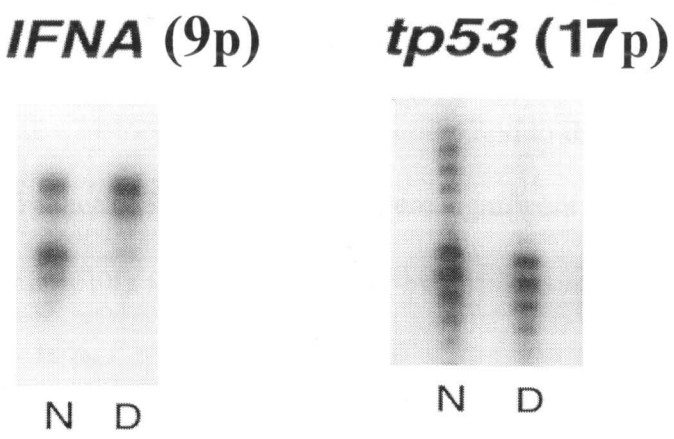
	Hyperplasia	Dysplasia	P value
# of lesions	25	42	
# with LOH	14/25 (56%)	26/42 (62%)	0.79
>1 arm lost	4/25 (16%)	17/42 (40%)	**0.06**
>2 arms lost	0/25 (0%)	7/42 (17%)	**0.04**
LOH on: 3p	6/25 (24%)	11/42 (26%)	1.0
9p	10/25 (4%)	21/42 (50%)	0.46
17p	2/25 (8%)	18/42 (43%)	**0.003**
3p &/or 9p	14/25 (56%)	23/42 (55%)	1.0
3p &/or 9p plus 17p	2/25 (8%)	15/42 (36%)	**0.02**

Table 13. LOH frequencies in hyperplasia, low- and high-grade dysplasias

	Hyperplasia	Low grade dysplasia	P[1]	High grade dysplasia	P [2]	P[3]
# of lesions	25	32		10		
# with LOH	14/25 (56%)	17/32 (53%)	1.0	9/10 (90%)	0.11	**0.06**
>1 arm lost	4/25 (16%)	8/32 (25%)	0.52	9/10 (90%)	**<0.0001**	**0.0004**
>2 arms lost	0/25 (0%)	1/32 (3%)	1.0	6/10 (60%)	**0.0001**	**0.0003**
LOH on: 3p	6/25 (24%)	4/32(13%)	0.31	7/10 (70%)	**0.020**	**0.001**
9p	10/25 (40%)	12/32 (38%)	1.0	9/10 (90%)	**0.0098**	**0.009**
17p	2/25 (8%)	10/32 (31%)	**0.05**	8/10 (80%)	**<0.0001**	**0.01**
3p &/or 9p	14/25 (56%)	13/32 (41%)	0.29	9/10 (90%)	0.11	**0.01**
3p &/or 9p plus 17p	2/25 (8%)	7/32 (22%)	0.27	8/10 (80%)	**<0.0001**	**0.002**

[1]Comparison between hyperplasia and low grade dysplasia. [2]Comparison between hyperplasia and high grade dysplasia. [3]Comparison between low-grade dysplasia and high-grade dysplasia.

Figure 6. Autoradiograph



Autoradiograph of DNA bands in a polyacrylamidegel. DNA isolated from normal tissue cells (N) has both the upper and lower bands. In contrast, the dysplastic cells (D) show only the upper band in the gel with *IFNA* primer and only the lower band in the gel with *tp53* primer, indicating chromosome loss at the region (loss of TSGs).

6.5. LOH and cancer history

LOH frequencies were analyzed with respect to the presence of history of head and neck cancer (Table 14). Consistently higher rates of LOH at all categories were observed in lesions from patients with a history of oral cancer as compared to those lesions from patients without a history of oral cancer. Such higher rates were significant for any loss (80% vs. 48% in patients without a history of oral cancer, $P = 0.01$), for >1 arm lost (48% vs. 21%, $P = 0.03$), at 17p (56% vs. 14%, $P = 0.0004$), for 3p and/or 9p (72% vs. 45%, $P = 0.04$) and for 3p and/or 9p plus 17p (48% vs. 12%, $P = 0.003$).

Table 14. LOH frequencies in patients with or without a history of oral cancer

	Without a history of oral cancer	With a history of oral cancer	P value
# of lesions	42	25	
# with LOH	20/42 (48%)	20/25 (80%)	**0.01**
>1 arm lost	9/42 (21%)	12/25 (48%)	**0.03**
>2 arms lost	3/42 (7%)	4/25 (16%)	0.41
LOH on: 3p	10/42 (24%)	7/25 (28%)	0.77
9p	16/42 (38%)	15/25 (60%)	0.13
17p	6/42 (14%)	14/25 (56%)	**0.0004**
3p &/or 9p	19/42 (45%)	18/25 (72%)	**0.04**
3p &/or 9p plus 17p	5/42 (12%)	12/25 (48%)	**0.003**

6.6. TB staining and LOH

In order to determine whether TB staining was associated with specific patterns of genetic alteration, irrespective of histology, LOH frequencies were compared between all TB positive lesions and negative lesions. As shown in Table 15, TB positive lesions had a significant increase in the following patterns compared to TB negative lesions: any loss (80% vs. 30%, $P = 0.0001$), > 1 arm lost (45% vs. 11%, $P = 0.004$), > 2 arms lost (18% vs. 0%, $P = 0.04$), and LOH at 3p (35% vs. 11%, $P = 0.04$), 9p (60% vs. 26%, $P = 0.007$), 17p (48% vs. 3%, $P = 0.0001$), 3p and/or 9p (73% vs. 30%, $P = 0.001$) and 3p and/or 9p plus 17p (40% vs. 4%, $P = 0.0006$).

In the latter analysis, both weak and strong staining lesions were scored as positive. In order to determine the significance of the weakly staining lesions with respect to molecular alterations, TB staining patterns were separately analyzed as negative, weakly positive and strongly positive (Table 15). When the TB negative cases were compared with weakly and strongly toluidine positive cases separately, significant differences still existed. Both weakly positive and strongly positive groups showed a higher rate of LOH compared to negative cases despite the smaller number of cases. Compared to the negative lesions, the weakly positive samples exhibited a higher rate of LOH for almost all categories with differences either significant or marginally significant for the following patterns: any loss (82% vs. 30%, $P = 0.0004$), > 2 arms lost (14% vs. 0%, $P = 0.08$), at 3p (36% vs. 11%, $P = 0.05$), at 17p (45% vs. 3%, $P = 0.001$), for 3p and/or 9p (68% vs. 30%, $P = 0.01$) and for 3p and/or 9p plus 17p (32% vs. 4%, $P = 0.02$).

Similarly the strongly positive samples, compared to negative samples, demonstrated a significant increase in the following patterns: any loss (78% vs. 30% in TB negative lesions, $P = 0.002$), > 1 arm lost (55% vs. 11%, $P = 0.002$), > 2 arms lost (22% vs. 0%, $P = 0.02$), LOH at 9p (72% vs. 26%, $P = 0.005$), 17p (50% vs. 3%, $P = 0.0004$), 3p and/or 9p (78% vs. 30%, $P = 0.002$) and for 3p and/or 9p plus 17p (50% vs. 4%, $P = 0.0004$).

In contrast, there was no significant difference in LOH frequencies between weakly positive and strongly positive lesions.

Table 15. LOH patterns in TB positive and negative samples

	TB Negative	TB positive (Weak + Strong)	P[1]	TB (Weakly positive)	P[2]	TB (Strongly Positive)	P[3]	P[4]
# of lesions	27	40		22		18		
# with LOH	8/27 (30%)	32/40 (80%)	**0.0001**	18/22 (82%)	**0.0004**	14/18 (78%)	**0.003**	1.0
>1 arm lost	3/27 (11%)	18/40 (45%)	**0.004**	6/22 (27%)	0.27	10/18 (55%)	**0.002**	0.11
>2 arms lost	0/27 (0%)	7/40 (18%)	**0.04**	3/22 (14%)	0.08	4/18 (22%)	**0.02**	0.68
LOH on: 3p	3/27 (11%)	14/40 (35%)	**0.04**	8/22 (36%)	**0.05**	6/18 (33%)	0.13	1.0
9p	7/27 (26%)	24/40 (60%)	**0.007**	11/22 (50%)	0.14	13/18 (72%)	**0.005**	0.20
17p	1/27 (3%)	19/40 (48%)	**0.0001**	10/22 (45%)	**0.001**	9/18 (50%)	**0.0004**	1.0
3p &/or 9p	8/27 (30%)	29/40 (73%)	**0.001**	15/22 (68%)	**0.01**	14/18 (78%)	**0.002**	0.72
3p &/or 9p plus 17p	1/27 (4%)	16/40 (40%)	**0.0006**	7/22 (32%)	**0.02**	9/18 (50%)	**0.0004**	0.33

[1]Comparison of patterns in TB negative and TB positive cases (includes weak and strong intensities). [2]Comparison of patterns in TB negative and TB weakly positive cases. [3]Comparison of patterns in TB negative and TB strongly positive cases. [4]Comparison of patterns in TB weakly positive and strongly positive cases.

6.7. TB staining and LOH pattern in hyperplasia and low-grade dysplasia

Since there were more high-grade dysplasia in the TB positive group (Table 9) and LOH frequencies are higher in such lesions compared to hyperplasia or low-grade dysplasia, it was possible that the elevated LOH frequencies reported above (Table 15) for TB positive lesions was a reflection of an alteration in the proportion of high-grade to low-grade (and non-dysplastic) lesions in the sample set. To rule out this possibility, LOH patterns were compared between TB positive and negative lesions with the sample set restricted to hyperplasia or low-grade dysplasia (Table 16). Compared to TB negative lesions, TB positive lesions still showed consistently higher frequencies of LOH for many of the aforementioned comparisons including the following: any LOH (71% vs. 31% in TB negative lesions, $P = 0.006$), LOH at 17p (36% vs. 4%, $P = 0.005$), LOH at 3p and/or 9p (61% vs. 31%, $P = 0.03$), and LOH at 3p and/or 9p plus 17p (25% vs. 4%, $P = 0.05$).

Table 16. LOH frequencies in TB positive and negative lesions that were histologically diagnosed as either hyperplasia or low grade dysplasia

	TB negative	TB positive	P value
# of lesions	26	28	
# with LOH	8/26 (31%)	20/28 (71%)	**0.006**
>1 arm lost	3/26 (12%)	8/28 (29%)	0.18
>2 arms lost	0/26 (0%)	1/28 (4%)	1.0
LOH on: 3p	3/26 (12%)	6/28 (21%)	0.47
9p	7/26 (27%)	13/28 (46%)	0.17
17p	1/26 (4%)	10/28 (36%)	**0.005**
3p &/or 9p	8/26 (31%)	17/28 (61%)	**0.03**
3p &/or 9p plus 17p	1/26 (4%)	7/28 (25%)	**0.05**

6.8. TB staining and LOH patterns in patients with or without a history of oral cancer

Since the TB positive group contained more lesions from patients with a history of oral cancer (Table 14), it was possible that the elevated LOH frequencies observed above (Table 15) for TB positive lesions was a reflection of an alteration in the proportion of lesions from cancer patients to primary lesions in the sample set. To rule out this possibility, LOH patterns were separately compared between TB positive and negative lesions in patients with and without a history of cancer (Table 17). In primary oral premalignant lesions, TB positive lesions still showed consistently higher frequencies of LOH for many of the

aforementioned comparisons including the following: any LOH (70% vs. 27% in TB negative lesions, $P = 0.01$), > 1 arm loss (35% vs. 9%, $P = 0.06$), > 2 arm loss (15% vs. 0%, $P = 0.10$), LOH at 9p (55% vs. 23%, $P = 0.06$), LOH at 17p (30% vs. 0%, $P = 0.007$), LOH at 3p and/or 9p (65% vs. 27%, $P = 0.03$), and LOH at 3p and/or 9p plus 17p (25% vs. 0%, $P = 0.02$).

In lesions from patients with a history of oral cancer, again, TB positive lesions showed higher LOH frequencies than negative lesions although the difference was only significant for any LOH (90% vs. 40%, $P = 0.04$). However, the lack of significance may be due to the small number of cases for patients with a cancer history.

Table 17. TB staining and LOH patterns in patients with or without a history of oral cancer

	Without a history of oral cancer			With a history of oral cancer		
	Toluidine negative	**Toluidine positive**	**P value**	**Toluidine negative**	**Toluidine positive**	**P value**
# of lesions	22	20		5	20	
# with LOH	6/22 (27%)	14/20 (70%)	**0.01**	2/5 (40 %)	18/20 (90%)	**0.04**
>1 arm lost	2/22 (9%)	7/20(35%)	0.06	1/5 (20%)	11/20 (55%)	0.32
>2 arms lost	0/22 (0%)	3/20 (15%)	0.10	0/5 (0%)	4/20 (20%)	0.55
LOH on: 3p	3/22 (14%)	7/20 (35%)	0.15	0/5 (0%)	7/20 (35%)	0.27
9p	5/22 (23%)	11/20 (55%)	**0.06**	2/5 (40%)	13/20 (65%)	0.36
17p	0/22 (0%)	6/20 (30%)	**0.007**	1/5(20%)	13/20 (65%)	0.13
3p &/or 9p	6/22 (27%)	13/20 (65%)	**0.03**	2/5 (40%)	16/20 (80%)	0.11
3p &/or 9p plus 17p	0/22 (0%)	5/20 (25%)	**0.02**	1/5 (20%)	11/20 (55%)	0.32

6.9. TB staining and LOH patterns in patients with multiple biopsies

In Table 18, biopsies taken from the same patient at either different sites or different times were evaluated for TB staining, histological diagnosis, treatment design and LOH patterns. Among 6 patients with multiple biopsies, 2 patients had negative TB staining at all times for their lesions (patients 1 and 2). The remaining 4 patients showed different staining results when a lesion was re-biopsied over a period of at least a year (patients 3-6). The uptake of TB dye by these lesions was associated with LOH for 3 of these patients: a reduction of TB staining from weakly positive to negative was accompanied by a reduction of allelic loss in

the lesion from LOH at 3 chromosome arms to 2 arms after bleomycin and laser treatment in patient #3; a reduction of TB staining from strongly positive to negative was accompanied by a reduction of allelic loss in the lesion from LOH at 3 chromosome arms to no loss after 2 rounds of bleomycin treatment in patient #4; an increase in TB staining from negative to weakly positive to strongly positive was accompanied by an increase of allelic loss in the lesion from LOH at 1 chromosome arm to 2 arms and then to 3 arms when no treatment was given in patient #5. For the last patient (#6), an increase in the uptake of the TB dye was associated with a persistence of the molecular changes (LOH at all 3 arms) and with histological progression of the lesion from hyperplasia to moderate dysplasia. These data support the use of the dye to predict outcome for a lesion.

Table 18. Results of patients with multiple biopsies

Patient#	Sample# (Biopsy time)	TB results	Diagnosis	Arms showing LOH	Treatment	Smoking Habit[1]	Cancer history
1	4[2] (1998)	Negative	Severe dysplasia	9p, 17p	Bleomycin once	N	No
	14[2] (1999)	Negative	Hyperplasia	9p, 17p		N	No
2	10[3] (1998)	Negative	Hyperplasia	No loss	No	S	Yes
	11[3] (1998)	Negative	Hyperplasia	No loss		S	Yes
3	19[4] (1997)	Weakly positive	Severe dysplasia	3p, 9p, 17p	Bleomycin once, followed by laser twice	N	Yes
	1[4] (1999)	Negative	Severe dysplasia	9p, 17p		N	Yes
4	36[5] (1996)	Strongly positive	Severe dysplasia	3p, 9p, 17p	Bleomycin twice	N	No
	6[5] (1998)	Negative	Mild dysplasia	No loss		N	No
5	7[6] (1989)	Negative	Mild dysplasia	9p	No	S	Yes
	27[6] (1993)	Weakly positive	Hyperplasia	9p, 17p	No	S	Yes
	34[6] (1995)	Strongly positive	Moderate dysplasia	3p, 9p, 17p		S	Yes
6	23[7] (1998)	Weakly positive	Hyperplasia	3p, 9p, 17p	No	S	Yes
	35[7] (1999)	Strongly positive	Moderate dysplasia	3p, 9p, 17p		S	Yes

[1]Smoking Habits: S = smoker; N = non-smoker; X = missing information. [2]Samples #4 and #14 were from the same lesion taken at different time (#4 in 1998 and #14 in 1999). [3]Samples #10 and #11 were different lesions taken at the same time from the same patient. [4]Samples #19 and #1 were from the same lesion taken at different time (#19 in 1997 and #1 in 1999). [5]Samples #36 and #6 were from the same lesion taken at different time (#36 in 1996 and #6 in 1998). [6]Samples #7, #27 and #34 were from the same lesion taken at different time (#7 in 1989, #27 in 1993, and #34 in 1995). [7]Samples #23 and #35 were from the same lesion taken at different time (#23 in 1998 and #35 in 1999).

6.10. Summary of results

In summary, the data from this study showed that compared to TB negative group, TB positive lesions were more frequently observed in older patients (p= 0,03), contained a significantly higher percentage of high-grade dysplasia (p= 0,03), contained a significantly higher percentage of lesions from patients with cancer history (p= 0.01), and contained a higher frequency of LOH (p= 0.0001) and high-risk LOH patterns (p= 0.0006). Also, the results from multiple biopsies showed that increased uptake of TB was associated with increased degree of dysplasia and increased LOH.

The increased LOH in TB positive lesions was independent of dysplasia and of cancer history.

From these results, it is suggested that TB can be used to identify high-risk OPLs.

7. DISCUSSION

One of the critical problems facing clinicians that deal with patients with OPLs is a determination of how to manage such lesions. Although the majority of OPLs are histologically diagnosed as either hyperplasia or low-grade dysplasia, only a small percentage will progress into cancer. Thus the development of markers that can be used to identify the more high-risk lesions is of vital importance. Recent data, some of which has come from this laboratory, support the use of molecular markers for risk prediction (Rosin *et al.*, 2000, 2002). However, both the more recent molecular markers and the traditional "gold standard" for risk prediction, histology, are largely dependent upon the decision of a clinician to biopsy an OPL and the choice of the biopsy site.

This thesis describes early results from an ongoing prospective study of 67 OPLs in which TB staining was evaluated for its ability to facilitate the identification of lesions with high risk of molecular progression. The preliminary results suggest that TB positive lesions have a higher cancer risk than TB negative lesions. As stated in the introduction, although TB is well documented as a visual aid for identification of early oral SCC and dysplasias where in experienced hands, this stain has been shown to have a high sensitivity (90% - 100%) for identifying oral SCCs, less consistent results with wide variations in sensitivity (42% - 75%) have been reported for oral dysplasias (see sections 1.7.6. and 1.7.7.). This thesis provides data that suggest that lesions that stain with this dye might have molecular patterns that are strongly associated with progression. As such, the thesis is one of very few studies that have looked for such an association (Guo *et al.*, 2001; Zhang et al., in press). The following is a

summation of the evidence from this thesis in support of the use of TB for risk assessment of OPLs.

7.1. TB positive lesions have increased cancer risk: support from histology

Consistent with the literature (see section 1.7.4.), this thesis showed that TB staining facilitated the identification of high-grade dysplastic OPLs, which are known to have high-risk for malignant transformation (see section 1.4.6.). Nine of 10 (90%) high-grade dysplasias were TB positive compared to 12 of 25 (48%) lesions histologically diagnosed as hyperplasia ($P = 0.03$). When the comparison group included both hyperplasia and low-grade dysplasia, 31 of 57 (54%) lesions were TB positive ($P = 0.04$ for comparison to high-grade lesions). These data support the use of TB as a visual aid to identifying high-grade OPLs.

7.2. TB positive lesions have increased cancer risk: support from cancer history

Leukoplakia in patients with a history of oral cancer have a much higher risk of progressing to cancer than leukoplakia in patients without such a history (Grant *et al.*, 1993). This thesis showed that there was a marked increase in the proportion of lesions that were TB positive among patients with a cancer history (see section 6.8. of results). Again, the results support the use of the dye to identify high-risk oral lesions.

7.3 TB positive lesions have increased cancer risk: support from LOH results

Recent studies from a number of laboratories have shown that high-risk OPLs are characterized by elevated LOH frequencies, most often on multiple arms (Califano et al., 1996; Mao et al., 1996; Rosin et al., 2000, 2002; Partridge et al., 2001). These studies have also shown that LOH at 3p and/or 9p is an early but essential step for oral carcinogenesis and for cancer progression, with additional losses at other arms markedly increasing risk. Of interest, LOH at 3p has been found to also occur in some cases as a late event, that is, a significant increase in 3p loss has been found in early oral carcinogenesis, with further loss noted with formation of cancer (Zhang *et al*, 1997; Poh *et al*, 2001; Guo *et al*, 2001). This may indicate that the loss of more than 1 locus on this chromosome arm is required for cancer development. On the other hand, LOH at 17 p and multiple losses are strongly associated with high-grade OPLs (severe dysplasia and *CIS*) and are usually felt to be late events in progression.

The most significant finding of this thesis is that TB positive lesions contained significantly increased LOH for all categories studied. This includes any loss, multiple losses (> 1 arm and > 2 arms lost), LOH at each individual chromosome arm examined (3p, 9p and 17p), and LOH for 3p &/or 9p, and for 3p &/or 9p plus 17p. The latter pattern represents the highest molecular risk pattern studied in this thesis. In a prior study from this laboratory, we reported the lowest progression risk for cases that retained both 3p and 9p. Risk was increased by 3.8-fold when either or both of these arms were lost. When loss at 3p and/or 9p was combined with LOH at 17p, the progression risk doubled yet again (above cases with

LOH at 3p and/or 9p only) (Rosin et al., 2000). Thus the strong associations of TB stain with the highest risk molecular pattern in this thesis strongly support the use of the stain to identify lesions at risk for progression.

The thesis has also demonstrated that TB staining is an independent predictor of risk. In other words, the association of TB stain with high-molecular risk patterns is not simply due to a greater proportion of high-grade dysplasia in the positively staining group. This was a concern that was addressed in the thesis, since, as stated above, TB positive lesions were more likely to be high-grade dysplastic lesions than TB-negative lesions. A comparison was also made of LOH patterns in TB positive and negative cases that were either hyperplasia or low-grade dysplasia (i.e. after exclusion of all high-grade lesions). The positive lesions showed significantly higher frequencies of loss for any LOH, and LOH at 17p, 3p &/or 9p and at 3p &/or 9p plus 17p.

We also tested the hypothesis that the increased frequency of high-risk molecular patterns in TB positive lesions was due to a greater proportion of patients with a history of oral cancer. When data was restricted to cases without a history of head and neck cancer, the association of LOH with TB positive stain still remained with a significantly higher frequency of LOH for most of the comparisons including any arm, multiple LOH (>1 arm and >2 arms lost), LOH at 9p, LOH at 17p, LOH at 3p &/or 9p and LOH at 3p &/or 9p plus 17p.

Our finding of an association between TB positive staining and high-risk LOH patterns (compared to TB negative lesions with similar histology) would suggest that TB stain is a

clinical risk factor independent of the presence and degree of dysplasia. Thus, the use of the dye would be supported for 2 reasons: because of its ability to identify dysplastic OPLs and, of equal importance, its ability to differentiate those OPLs with progression risk. Our results are in agreement with a recent publication from John's Hopkins. Guo *et al.* (2001) showed that among 25 patients with two biopsies of oral premalignant lesions (one from toluidine positive area, another from adjacent negative area), 16 had identical allelic losses. In the remaining 9 cases, eight showed LOH at more genetic loci in TB positive biopsies compared to the negative biopsies.

Another interesting finding of this study was that there were no significant difference in the molecular profiles of OPLs with strong TB staining compared with those with weak staining. Both showed significantly increased LOH in comparison to TB negative lesions. Weak staining is not uncommon in OPLs, and the interpretation of such stain is variable. Some call it positive, others equivocal, and still others negative. Our results suggest that one should carefully evaluate a weak TB stain. The results support the practice of using TB to delineate a lesion even when such staining is faint.

7.4. TB positive lesions have increased cancer risk: support from multiple biopsy results

Our data from patients with multiple biopsies also supports an association between TB staining and an increased cancer risk. We observed an alteration in TB staining in repeat biopsies of 4 of the 6 patients with multiple biopsies examined in this study (Table 18). In 2

of the 4 lesions, treatment between repeat biopsies resulted in a reduction in TB staining that was accompanied by decrease in the number of genetic loci showing LOH. For the 2 untreated lesions, increases in the TB staining of the lesions were accompanied by an increase in allelic loss in one lesion (from '9p' → '9p and 17p' → '3p, 9p and 17p'). In the other, persistence of LOH at all 3 arms assayed and an increase in TB staining was associated with progression of the lesion histologically from hyperplasia to moderate dysplasia. Such results suggest that an increased uptake of the toluidine dye in a lesion is associated with an increase in molecular or histological risk in a lesion.

7.5. Comments on the association of LOH with histological diagnosis

Although high frequencies of LOH occurring at multiple loss are most often seen in later histological stages, several reports suggest that this association is not always true (Califano et al., 1996; Mao et al., 1996; Rosin et al., 2000, 2002; Partridge et al., 2001). Some low-grade dysplasia and hyperplasias also have elevated risk patterns. In my thesis, high-grade dysplastic lesions had significantly increased frequencies of LOH compared to non-dysplastic lesions or low-grade dysplasias including an increase in the presence of multiple LOH (> 1 arm lost and > 2 arm lost), and in LOH at each of the individual chromosome arms examined (3p, 9p and 17p). Also of significance is the fact these high-grade dysplasias had a significant increase in the proportion of cases with 3p and/or 9p plus 17p LOH. Nine of 10 high-grade lesions contained LOH at 3p and/or 9p (required for cancer progression) and 8/10 high-grade lesions had 3p and/or 9p plus 17p. The latter pattern represents the highest molecular risk pattern studied in this thesis.

Unlike the previous study from this lab, which showed very low allelic loss in hyperplastic lesions (Rosin et al., 2000), this thesis showed that LOH frequencies in hyperplasia were not significantly different from those of low-grade dysplasia. There is, however, a major difference in the sample populations between the previous study and the current study. The hyperplasias used in the previous study were reactive or inflammatory lesions (including mucocele, periodontitis, fibroepithelial hyperplasia) from patients without a history of oral cancer. These hyperplastic lesions have no known cancer risk and are not premalignant lesions, and they were used in the previous study as a control to rule out the possibility that allelic loss was associated with cell proliferation instead of true premalignancy. The hyperplastic lesions used in this study were clinically leukoplakia, which even in the absence of dysplasia may have some low cancer risk. Furthermore, some of the hyperplastic lesions (7/25) in this thesis were from patients with a history of oral cancer, which again designates increased cancer risk. Such results are interesting and clinically significant as it again points to the limitation of histology, the current gold standard for malignant risk evaluation.

A further support for the use of LOH to predict risk comes from a comparison of LOH frequencies in leukoplakia from patients with a cancer history to patients with no prior history. The data from this thesis showed that a greater proportion of lesions from patients with a history of oral cancer had LOH. This included an increased frequency of cases with multiple losses and with LOH at 3p and/or 9p plus 17, the high-risk pattern.

7.6. Mechanism for selective staining by TB of high-risk OPLs

Why does TB preferentially stain OPLs with high-risk molecular profiles? The mechanisms underlying this stain are unknown. TB is a basic thiazine metachromatic dye with affinity for acidic components of tissues and hence tends to bind to DNA and RNA (Martin *et al*, 1998). Hypotheses for toluidine staining of SCCs and OPLs include increased DNA and RNA of lesions, especially in the superficial cell layers, and defective barriers of the epithelial cells, which allow the dye to penetrate to deeper cellular layers with higher DNA and RNA concentrations (Dunipace et al, 1992; Strong *et al*, 1968). The general strong staining of almost all SCCs can be explained by the fact that the above abnormalities are much more pronounced in SCC compared to premalignant lesions. Similarly high-grade OPLs are more likely to stain with TB than low-grade lesions as abnormalities mentioned above are probably more pronounced. In the same way, OPLs with high-risk molecular profiles could have higher cellular content of DNA and RNA, and more defective cell barriers allowing penetration of the dye, hence they are more likely to be positive, compared to OPLs with low-risk molecular profiles.

Supporting this hypothesis is a recent study which found an association of ploidy in dysplastic lesions with progression risk for OPL (Sudbo *et al.*, 2001). The lowest risk was associated with diploid dysplastic leukoplakias (3% progressed into oral SCC), tetraploid lesions had an intermediate-risk (60% showed progression), and aneuploid dysplasias showed the greatest risk (84% progressed). This suggests that high-risk OPLs have

increased chromosome content compared to morphologically similar low-risk lesions, and that this alteration could be responsible for the increased uptake of toluidine dye.

7.7. Summary

In summary, this study showed that TB positive lesions tend to contain high-risk molecular profiles. Currently we are verifying the results by increasing sample size of the study groups, by increasing the number of primers for LOH analysis and by correlating the toluidine study results with the outcome of the lesions. If the study results are further confirmed, it may have important clinical implications. It would suggest that TB stain could be used to not only identify dysplastic OPLs, but that it might also serve as an independent risk predictors for such lesions. The dye could alert clinicians of cancer progression risk in low-grade OPLs or in premalignant lesions without dysplasia. The latter are lesions in which the current gold standard, histology, has a poor predictive value for cancer risk. Similarly, the dye could be used as an adjunct technique to help identify small, asymptomatic lesions, multiple lesions and high-risk areas that need to be biopsied. All of these approaches could lead to improvements in the prognosis and management of oral SCC.

8. APPENDIX

Appendix 1: Clinicopathological and molecular data for individual lesions

Tube ID	Cancer History	Histological Diagnosis	Site	Smoking habit	Gender	Age	Any LOH	Molecular risk	3p T	9p T	17p T
630H1-2	0	H	Lat Tong	0	M	66	1	2	L	L	R
535D3	0	D1	Lat Tong	0	M	49	1	2	L	L	R
535D4	0	D1	Lat Tong	0	M	49	1	2	R	L	R
535D2	0	D3	Lat Tong	0	M	49	1	2	L	L	R
618D1-2	0	D1	Gingiva	1	F	75	0	1	R	R	R
618H1-1	0	H	Gingiva	1	F	75	0	1	R	R	R
545D1-1	1	D1	Lat Tong	0	F	74	1	3	R	L	L
545D2-2	1	D1	Lat Tong	0	F	75	0	1	R	R	R
545D4-1	1	D1	Lat Tong	0	F	75	1	1	R	R	L
545D4-2	1	D1	Lat Tong	0	F	75	1	3	R	L	L
545D1-2	1	D3	Lat Tong	0	F	74	1	3	R	L	L
545H2-1	1	H	Lat Tong	0	F	75	1	3	R	L	L
634D1	0	D1	Soft Palate	0	F	43	0	1	R	R	R
377H4-1	1	H	L Corner of mouth	1	M	56	1	2	L	R	R
377H4-2	1	H	Cheek	1	M	56	1	2	L	R	R
359H4	0	H	Cheek	1	M	63	1	2	L	R	R
359H7	0	VH	Cheek	1	M	65	1	2	L	R	R
365D2-1	1	D1	Gum	1	F	81	1	3	R	L	L
365D2-2	1	D2	Cheek	1	F	81	1	2	R	L	R
365H1	1	VH	Gum	1	F	80	1	2	R	L	R
239D2	0	D1	Vestibule	1	M	93	0	1	R	R	R
239H2	0	H	Vestibule	1	M	93	1	2	R	L	R
635H1-1	0	H	Lip	1	M	40	0	1	R	R	R
635H1-2	0	H	Gum	1	M	40	0	1	R	R	R
383D	1	D3	FOM	1	M	56	1	3	L	L	L

Tube ID	Cancer History	Histological Diagnosis	Site	Smoking habit	Gender	Age	Any LOH	Molecular risk	3p T	9p T	17p T
380D	1	D3	Retomolar Pad	1	F	68	1	3	L	L	L
470T2	1	D3	Lip	1	M	47	0	1	R	R	R
615H3	1	VH	Lat Tong	1	F	56	1	3	R	L	L
509H	0	H	Lat Tong	1	F	84	1	2	R	L	R
626H1	0	H	Lat Tong	1	M	34	0	1	R	R	R
363D5	1	D3	Lat Tong	1	F	77	1	3	L	L	L
210D6	0	D1	Lat Tong	0	F	40	0	1	R	R	R
210D1-2	0	D3	Lat Tong	0	F	38	1	3	R	L	L
210D1-1	0	D3	Lat Tong	0	F	38	1	3	L	L	L
624H1-2	0	H	Gum	0	M	58	0	1	R	L?	R
632D1	0	D2	Lat Tong	0	F	60	1	2	NI	L	R
301D	0	D2	Vestibule	1	F	67	0	1	R	L?	R
518H	0	H	Cheek	1	M	56	0	1	R	R	R
418D1B	0	D1	FOM	0	M	83	1	3	R	L	L
418D1A	0	D2	Lat Tong	0	M	83	1	1	R	R	L
288H2	0	H	Hard Palate	1	M	69	0	1	R	R	R
562D4	0	D1	Hard Palate	1	F	64	0	1	R	R	R
562D2	0	D3	Palate	1	F	64	1	3	L	L	L
562D1	0	D2	Cheek	1	F	64	1	3	L	L	L
238D3	1	D1	Lat Tong	0	M	91	1	1	R	R	L
238D2	1	D3	Lat Tong	0	M	90	1	3	L	L	L
633H2-1	1	D1	Lat Tong	1	F	52	1	2	IC	L	R
633H1-2	1	H	Lat Tong	1	F	52	0	1	R	L?	R
616D5	1	D2	Lat Tong	1	M	59	1	3	L	R	L
636D1-1	0	D1	Alveolar Ridge	0	F	73	0	1	R	R	R
622H1-2	0	H	Gingiva	0	F	58	0	1	R	R	R
622H1-1	0	H	Gingiva	0	F	58	1	2	L	L	R
629D2-2	0	D1	Gingiva	0	F	60	0	1	R	R	R
629H2-1	0	H	Gingiva	0	F	60	0	1	R	R	R
623H1	0	H	FOM	1	F	41	1	2	R	L	R
617H4	1	H	Vestibule	1	F	52	1	2	R	L	R
703D1	1	D1	Lat Tong	1	M	83	1	3	R	L	L

Tube ID	Cancer History	Histological Diagnosis	Site	Smoking habit	Gender	Age	Any LOH	Molecular risk	3p T	9p T	17p T
682D1	0	D2	FOM	1	F	40	1	2	L	R	R
681D1	0	D1	Lat Tong	1	F	71	1	2	R	L	R
685D1	0	D1	Soft Palate	1	M	50	0	1	R	R	IC
686D1	0	D2	Soft Palate	1	M	65	0	1	R	R	R
688D1	0	D2	Lat Tong	1	F	62	0	1	R	L?	R
700D1	0	D2	Gum	0	F	41	0	1	R	R	R
697D1-1	1	D2	Lat Tong	0	F	40	0	1	L?	L?	IC
704D1-3	0	D1	FOM	1	F	61	0	1	IC	R	IC
704H1-2	0	H	Cheek	1	F	61	0	1	IC	R	R
704H1-1	0	H	Cheek	1	F	61	1	2	R	L	IC

Cancer History: 0 = without a history of Head and Nedk Cancer; 1 = with a history
Histological Diagnosis: H = hyperplasia; VH =verrucous hyperplasia; D1 = mild dysplasia; D 2= moderate dysplasia; D3 = severe dysplasia
Site: Lat Tong = lateral tongue; L corner of mouth = left corner of mouth; FOM = floor of mouth
Smoking Habit: 0 = no; 1 = yes for ever smoker
Gender: F = female; M = male
Any LOH: 0 = no; 1 = yes
Molecular Risk Pattern: 1 = Retention of both 3p & 9p; 2 = LOH at 3p &/or 9p; 3 = LOH at 3p &/or 9p plus 17p
3p-T = LOH on at least 1 loci for chromosome 3; 9p-T = LOH on at least 1 loci for chromosome 9; 17p = LOH on at least 1 loci for chromosome 17

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