USE OF TISSUE AUTOFLUORESCENCE IN THE DETECTION OF ORAL PREMALIGNANT LESIONS WITH HIGH-RISK MOLECULAR PATTERNS

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ABSTRACT

There is a pressing need for the development of visual aids to help identify oral premalignant lesions (OPLs) at risk of cancer progression. This thesis investigated whether OPLs could be identified by fluorescence visualization (FV), a simple hand-held device developed by our research team. Ninety-one oral lesions were judged by FV as showing normal fluorescence (FV retained, FVR) or FV loss (FVL). FVR and FVL lesions were assessed and compared histologically, molecularly (analysis for loss of heterozygosity, LOH, on 3p, 9p, 4q, 8p, 11q, 13q, and 17p) and by outcome. The results showed a strong association of FVL with pathologic risk (high-grade dysplasia, $P <$ 0.0001), with high-risk molecular patterns ($P = 0.02$ for multiple loss; $P = 0.004$ for lesions with LOH at 3p &/or 9p), and with cancer progression ($P = 0.07$). In summary, FV may serve as an adjunct tool to assist clinicians in identifying high-risk OPLs.

EXECUTIVE SUMMARY

Background: The assessment of cancer risk for oral premalignant lesions (OPLs) is currently based mainly upon histological diagnosis, and more recently, molecular studies. For example studies from our research team have found that loss of heterozygosity (LOH) pattern could classify morphologically indistinguishable OPL with minimal (mild/moderate) or no dysplasias into different risk categories: Compared with OPLs without LOH at 3p and 9p, those with LOH at 3p and/or 9p but not in other arms had a 3.8-fold increase in relative cancer risk, and those with LOH at 3p and/or 9p plus additional losses (on 4q, 8p, 11q, or 17p) had a 33-fold increase in relative cancer risk. However both histololgy and molecular studies largely depend upon tissue biopsies. The decision of when and where to biopsy in turn relies heavily on the clinician's ability to identify high-risk lesions or high-risk areas of lesions needing biopsies. Clinical diagnoses and differential diagnoses of OPLs/early cancer are difficult, and this frequently results in delay of biopsy. Furthermore, a significant percentage of OPLs and early cancer are not clinically apparent or visible. Visual tools that could help clinicians to decide when and where to biopsy high-risk oral lesions are highly desired. Fluorescence visualization (FV), a simple hand-held device developed by our research team., is designed to study tissue autofluorescence in the oral cavity.

Objective: To determine whether FV can identify OPLs at risk of cancer progression as judged pathologically, molecularly and by outcome.

Methods: The study population involves consented patients (with a history of oral cancer or dysplasia) enrolled in the Oral Dysplasia Clinic at BC Cancer Agency for a longitudinal study. The oral lesions of the patients were judged by FV as showing normal fluorescence (fluorescence visualization retained, FVR) or FV loss (FVL). FVR and FVL lesions were assessed and compared histologically, molecularly (microsatellite analyis for LOH on 3p, 9p, 4q, 8p, 11q, 13q, and 17p) and by outcome.

Results: Of the 91 oral lesions examined with FV, 56 showed FVL and 35 FVR. FVL was strongly associated with pathologic risk (high-grade dysplasia, $P <$ 0.0001), with high-risk molecular patterns ($P = 0.02$ for multiple loss and $P = 0.004$ for lesions with LOH at 3p &/or 9p), and with cancer progression ($P = 0.07$).

Conclusion: These data support the potential utility of FV as an adjunct tool to assist the clinician in identifying oral lesions requiring more intensive treatments and follow-up.

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LIST OF ABBREVIATIONS

BCCA:	British Columbia Cancer Agency
CDK:	Cyclin-dependent kinase
CIS:	Carcinoma *in situ*
DNA:	Deoxyribonucleic acid
FAD:	Flavin adenine dinucleotide
FV:	Fluorescence Visualization
H&E:	Hematoxylin and eosin
HNSCC:	Head and neck squamous cell carcinoma
HPV:	Human Papilloma Virus
LOH:	Loss of heterozygosity
NADH:	Nicotinamide adenine dinucleotide (reduced form)
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
OPL:	Oral premalignant lesion
PC:	Phenol-chloroform
PCR:	Polymerase chain reaction
PEP:	Primer-extension preamplification
RFLP:	Restriction fragment length polymorphism
SCC:	Squamous cell carcinoma
SDS:	Sodium dodecyl sulfate
SFU:	Simon Fraser University
SILS	squamous intraepithelial lesion site
STR:	Short tandem repeat
TB:	Toluidine blue
TSG:	Tumour suppressor gene
UV:	Ultraviolet
WHO:	World Health Organization

I. INTRODUCTION

I.1. Overview of oral cancer

More than 300,000 new cases of oral squamous carcinoma are diagnosed worldwide and 130,000 people die of the disease annually. The area with the highest incidence is Melanesia (31.5 per 100,000 in men and 20.2 per 100,000 in women). Rates in men are high in Western Europe (11.3 per 100,000), Southern Europe (9.2 per 100,000), South Asia (12.7 per 100,000), Southern Africa (11.1 per 100,000), and Australia/New Zealand (10.2 per 100,000). In females, incidence is relatively high in southern Asia (8.3 per 100,000). These patterns reflect prevalence of specific risk factors, such as tobacco/alcohol use in Western Europe, Southern Europe, and Southern Africa, and the chewing of betel quid in south central Asia and Melanesia (Parkin *et al.* 2005). In the United States approximately 30,000 new cases are diagnosed annually, and 8,000 people die each year (Jemal *et al.* 2005). In Canada, about 3,200 new cases of oral cancer arise every year, and 900 of these result in death (National Cancer Institute of Canada 2004).

Oral cancer has one of the lowest survival rates among the major human cancers. The 5-year survival rate remains about 50% despite advances in treatment with surgery, radiation and chemotherapy. This poor prognosis for oral cancer has not improved significantly over the last 4 decades largely due to the late diagnosis of the disease (Jemal

et al. 2005; Lee *et al.* 2000). While early stage oral cancer (I and II) has a five-year survival rate of 80%, the late stages (III and IV) have only a 20~30% five-year survival rate. Early diagnosis, particularly diagnosis of the lesions at the premalignant stage, is critical for improving the dismal prognosis. One of the major obstacles in the early diagnosis of the high-risk lesions, including both oral premalignant lesions (OPLs) and early cancer, is the difficulty in the clinical diagnosis of these lesions. Development of visual tools, which could help the clinicians in the decision of when and where to biopsy oral lesions, is highly desired. This study involves validation of new visual tool fluorescence visualization (FV), a simple hand-held device that detects changes of autofluorescence of oral mucosa.

In the following sections, I will review the risk factors for the development of oral cancer, the histology of oral mucosa, current clinicopathological parameters used for the diagnosis of high-risk oral lesions, recent advances in molecular markers for the prediction of cancer risk of OPLs, and the use of Fluorescence Visualization (FV) in the identification of cancer and precancerous lesions.

I.2. Etiological factors for oral cancer

Oral cancer has multiple etiological factors, and it is generally accepted that cancer develops as a result of the interaction of numerous factors, some environmental and others genetic. Tobacco exposure, alcohol consumption, papilloma virus infection, diet, and the presence of specific genetic polymorphisms have all been identified as risk factors for the development of oral cancer (Zavras *et al.* 2001; Znaor *et al.* 2003). But the major risk factors are tobacco use and alcohol consumption (Blot *et al.* 1988; Ogden

2005; Warnakulasuriya *et al.* 2005). The potential roles of each of these factors will be discussed below.

I.2.1. Tobacco

Evidence from many epidemiology studies has provided documentation of the association between tobacco use and oral cancer (Doll 1950). The risk for oral cancer in cigarette smokers is substantially greater than that observed in people who have never smoked. Most studies have reported mortality ratios for smokers versus non-smokers of about 5~6:1, with several reporting ratios in excess of 10:1. Furthermore, the risk for death from oral cancer is consumption related; the more cigarettes consumed daily and the more years one has smoked, the greater the risk. (US Dept. of Health and Human Services, 1982, 1989).

The oral use of smokeless tobacco also represents a significant health risk. It is not a safe substitute for smoking cigarettes, because it can cause cancer and a number of non-cancerous oral conditions and lead to nicotine addiction and dependence. Especially in India, chewing tobacco is believed to be responsible for the high incidence of oral cancer (Marshall *et al.* 1992; Rodu and Jansson 2004).

The majority of carcinogens in tobacco smoke are the by-products of pyrolysis (White *et al.* 2001). Chemical analysis reveals that smoke from a single cigarette is composed of over 6,000 different constituents, including some that are pharmacologically active, toxic, mutagenic, or carcinogenic (US Dept. of Health and Human Services, 1982, 1989). Some of the carcinogens include polycyclic aromatic hydrocarbons, aromatic amines, N-nitrosamines, and tobacco specific N-nitrosamines (Hoffmann *et al.* 1997).

Smokeless tobacco also contains carcinogens like tobacco-specific N- nitrosamines, some at extremely levels (Hoffmann *et al.* 1994).

I.2.2. Alcohol

Studies that have found alcohol use to be a risk factor for oral carcinogenesis usually conclude that the level of consumption is important (Andre *et al.* 1995; Znaor *et al.* 2003). Usually heavy drinkers are also heavy smokers. Studies have shown that, used in combination, alcohol and tobacco exert a synergistic effect that substantially increases the risk for oral cancer (Franceschi *et al.* 1990). A metabolite, acetaldehyde, may play a major role (Zavras *et al.* 2002). Alcohol may also act as a co-carcinogen to increase the penetration of carcinogens found in tobacco through the epithelium, acting as a solvent.

I.2.3. Diet

There is strong evidence for the inadequacy of many dietary components in cancer development. For example, consuming food rich in fibers, vitamins and phytochemicals have all been reported to show protective effects on cancer development at many sites. More specific, studies have reported that a diet low in beta-carotene, vitamin C, vitamin E have been associated with an increased risk of oral cancers (Block 1991; Mirvish 1986; Wald 1987).

I.2.4. Viruses

Extensive epidemiologic and laboratory evidence supports a role for infection with Human Papilloma Virus (HPV) in oral cancers, although the risk seems to be more

strongly associated with oropharyngeal cancer. Based on current estimate, about 18% of the oral cancer and 50~70% cancer of the oropharyngeal region (back of the tongue and tonsillar area) are associated with HPV infection (Gillison *et al.* 1999; Gillison and Shah 2001).

HPV appears to act primarily by inactivating two critical control proteins in the cell: p53 and retinoblastoma (Rb) with this regulation involving 2 viral proteins produced by the virus E6 and E7 respectively (Das and Nagpal 2002; Herrero *et al.* 2003). Loss of p53, in turn, removes the central control of cell proliferation, DNA repair, apoptosis and angiogenesis. The *Rb* gene controls the entry of cells into the cell cycle past the restriction points (Alberts *et al.* 1994). There are still substantial gaps in our knowledge about the interaction of HPV and other viruses in the development of oral cancer (Vambutas *et al.* 1993; Woods *et al.* 1993).

In summary, the most significant known etiological factors for oral cancer are tobacco and alcohol. Evidence for the relationship of such habits to oral cancer risk is extensive. Epidemiological evidence indicates that it generally takes decades of exposure to tobacco and alcohol for the development of precancerous and cancerous lesions. The length of the process allows early intervention if carcinogenesis can be identified.

I.3. Anatomy and histology of normal oral mucosa

The oral cavity is the beginning of the gastrointestinal system, functioning physically in the ingestion, fragmentation, moistening and the beginning of the digestion of food. In addition, it has some very important additional functions such as speech,

facial expression, sensory expression, and breathing. The oral cavity includes many parts: the lips, the lining inside the lips and cheeks (the buccal mucosa), the teeth, the bottom (floor) of the mouth under the tongue, the front two-thirds of the tongue, the bony top of the mouth (hard palate), the gums, and the small area behind the wisdom teeth. The oropharynx includes the back one-third of the tongue, the soft palate, the tonsils, and the part of the throat behind the mouth. Salivary glands throughout the oral cavity make saliva, which keeps the mouth moist and helps digesting food.

The oral cavity is lined with a stratified squamous epithelium and a lamina propria consisting mostly of connective tissue. The basement membrane is the physical barrier between the overlying epithelium and the underlying connective tissue. This membrane has two distinct layers: the basal lamina, immediately adjacent to the basal cells, produced by the epithelial cells and containing collagen type IV; and the reticular lamina, produced by fibroblasts of the underlying connective tissue and containing fibrillar collagen(Burkitt 1993).

The overlying stratified squamous epithelium contains four cell layers: the stratum germinativum (stratum basale or basal cell layer), the stratum spinosum (prickle cell layer), the stratum granulosum (granular layer), and stratum corneum (cornified layer). The different layers of the oral epithelium represent a progressive maturation process. The cuboidal-shaped basal cells form a single-cell layer that exists between the epithelium and connective tissue. The cells within this basal cell layer contain progenitor cells that have the capacity to divide and give rise to more new basal cells or differentiate into prickle cells. As the cells mature, they migrate toward the surface, changing their

shape into more elongated and flattened forms. Once reaching the surface, they are eventually desquamated (Berkovitz and Pacy 2002; Burkitt 1993).

The cell layer components of the stratified squamous epithelium vary in different oral sites. While stratified squamous epithelium always contains the basal and prickle cell layers, the epithelium in some parts of the oral cavity lacks the granular and cornified layers when the lining is not keratinized. The mucosa of the cheeks, lips, alveolus, floor of mouth, ventral surface of tongue, and soft palate is of this kind; it has a loose lamina propria, and is referred to as lining mucosa. However, some oral epithelium regions susceptible to mechanical forces such as gingiva and hard plate are characterized by a keratinized or parakeratinized epithelium and a thick lamina propria, and are referred to as the masticatory mucosa. This mucosa serves as a very effective mechanical and permeability barrier. Lastly, the dorsum of the tongue is composed of a specialized epithelium that is a mixture of both nonkeratinized and keratinized tissues, which are attached tightly to the underlying tongue muscle. It is referred to as the gustatory mucosa (Berkovitz and Pacy 2002; Burkitt 1993).

The underlying connective tissue holds blood and lymphatic vessels, nerves, and muscle fibres. This basic pattern of an epithelium and supporting connective tissue is analogous to the pattern of the epidermis and dermis of the skin. The main bulk of the connective tissue comprises both fine and coarse collagen. In normal tissue, collagen fibres are thin and loosely arranged in the superficial papillary layer while the deep reticular layer is dominated by thick, parallel bundles of collagen fibres. Elastin is another constituent that forms a fine interlacing network of fibres which follow the path of the interlacing collagen bundles.

Over 90% of oral malignancies, excluding salivary gland tumours, develop from this stratified squamous epithelial tissue; hence the name squamous cell carcinoma (SCC) (Shiboski *et al.* 2000).

I.4. Oral premalignant lesions (OPLs)

In 1978, the World Health Organization (WHO) 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". The majority of OPLs do not become cancer.

In the oral cavity, the most common premalignancy is leukoplakia. The term leukoplakia describes a white patch or plaque that does not rub off and cannot be clinically and pathologically identified as other disease and has increased cancer risk (WHO, 1978). The less common clinical presentation of OPLs is erythroplakia, defined as "a lesion that presents as a red area and cannot be diagnosed as any other definable lesion" (Axell *et al.* 1996). Erythroplakia has a much greater probability of showing signs of either invasive carcinoma, carcinoma *in situ,* or severe epithelial dysplasia at the time of diagnosis (Shafer and Waldron 1975). The malignant potential of lichen planus, another relatively common lesion, is in dispute.

I.5. Clinicopathological factors in prediction of oral cancer development

As mentioned above, OPLs only have an increased likelihood of cancer, and the majority of premalignant lesions do not become cancerous. A number of clinicopathological features including morphological alterations of OPLs are known to have predictive value in the malignant risk assessment of OPLs. However, these parameters are far from adequate as risk predictors and our current ability to predict which OPLs will undergo malignant transformation is quite limited. The following sections discuss these risk factors and their limitations.

I.5.1. Clinical features of OPLs

Current clinical diagnoses of OPLs involve clinical identification of OPLs based on the clinical risk factors. There are four main clinical risk factors: clinical appearance, site and size of OPLs, and history of head and neck cancer. These will be discussed below along with some other risk factors.

I.5.1.1. Clinical appearance

In the oral cavity, OPLs mostly present as leukoplakia and only occasionally as erythroplakia. Since the majority of OPLs are leukoplakia, the term leukoplakia has been used interchangeably with the term OPL, which includes both leukoplakia and erythroplakia.

Leukoplakia can be classified into two major types according to their clinical appearance. The most common form, called homogeneous leukoplakia, refers to leukoplakia that is homogeneous both in colour and texture. They are predominantly white or slightly yellowish and have a smooth, sometimes slightly wrinkled texture. The other type, referred to as non-homogeneous leukoplakia, include leukoplakias that are non-homogeneous either in colour or texture or both. They could have one or more of the following characteristics: a rough surface (nodular or verrucous), speckles of red patches (speckled leukoplakia), or erythroplakia.

There is strong evidence that non-homogeneous leukoplakia have a higher cancer risk than homogeneous leukoplakia and many erythroplakia are already cancer (Axell *et al.* 1996; Pindborg *et al.* 1968; Rajendran *et al.* 1989; Shafer and Waldron 1975). Because of the strong correlation between clinical appearance (homogenous vs. non-homogenous) and the cancer risk of OPLs, many staging systems for cancer risk of OPLs include the criteria of clinical appearance (Axell *et al.* 1996; Schepman and van der Waal 1995).

The clinical appearance of OPLs can help to predict cancer risk particularly in those cases with either obvious nodular/verrucous leukoplakia or erythroplakia. However, clinical appearance is frequently inadequate in assessing the cancer risk of OPLs for the following reasons: (1) OPLs are not always clinically visible; (2) sometime even the most innocuous homogeneous leukoplakia could turn out to be *CIS*; (3) it is hard to differentiate between non-homogeneous lesions with less striking appearance and homogeneous leukoplakia with a slightly rough surface; and (4) many reactive lesions are similar to homogeneous leukoplakia (Schepman *et al.* 1999).

I.5.1.2. Site of the OPLs

The oral cavity can be divided into high- and low-risk sites. The high-risk sites within the oral cavity for countries in the Western world are the floor of mouth (FOM), ventrolateral tongue, and soft palate–anterior pillar–retromolar complex (soft palate complex) (Shiboski *et al.* 2000). The rest of the oral cavity is designated as low-risk.

There is strong evidence that site is a high-risk predicting factor, and it has been included as one of the risk predictors in three proposed staging systems for OPLs (Axell *et al.* 1996; Pindborg *et al.* 1968; Schepman and van der Waal 1995). Epidemiological investigations in the Western part of the world have consistently shown that cancerous and pre-cancerous lesions mostly reside in high-risk sites (Mashberg and Meyers 1976; Waldron and Shafer 1975). Dysplastic lesions in high-risk sites are also more likely to progress into cancer than those located in low-risk sites with similar degree of dysplasia (Kramer *et al.* 1978; Zhang and Rosin 2001).

Though the 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. One popular theory proposes that high-risk sites are generally located in the lower part of the oral cavity, where the mucosae generally have prolonged exposure to carcinogens. Another theory is that keratinization in many of the low-risk sites might prevent carcinogen penetration to the basal cells, where the dividing cells lie. In contrast, high-risk sites are usually covered by a fragile, thin squamous epithelium.

Despite the high rate of malignant transformation for OPLs in the high-risk sites, many of OPLs there still do not progress. Likewise, a small percentage of lesions at low-risk sites do progress cancer. Tools that could help clinicians to further triage cancer risk of OPLs are needed.

I.5.1.3. Size of the lesion

It is generally believed that the bigger the premalignant lesion a patient has, the higher the cancer risk will be. The concept of size here refers to the combination of all leukoplakias in the oral cavity if there is more than one. The actual risk value of the size of OPLs is still unclear. The cut-off size for different risks remains speculative. In four proposed staging system for OPLs, it has been proposed that judging by size alone, an OPL with a dimension less than 2 cm has the lowest cancer risk, 2–4 cm has intermediate risk, and larger than 4 cm has high cancer risk (Axell *et al.* 1996; Pindborg *et al.* 1968; van der Waal *et al.* 2000). Most oral lesions fall into the category of less than 2 cm and are deemed to have a low cancer risk.

I.5.1.4. Duration of the lesion

If we discuss size with cancer risk, we must also discuss the duration of the lesion. Not only is size important as a risk factor, but the change in size over time is also important (Pindborg *et al.* 1968). Generally, a lesion that decreases in size or disappears completely over time (after the withdrawal of a carcinogen, e. g. quitting smoking) has a much lower risk than a similar sized lesion that is stagnant or even increases in size or persists.

In other words, the longer a lesion persists (remains the same size or increases in size), the higher the cancer risk. Conceptually, this makes sense since older lesions have a longer time to acquire additional genetic and histological changes than younger lesions. However, the literature varies in the reported time between initial identification of leukoplakia and diagnosis of cancer (duration of OPLs). Some report that most carcinoma with leukoplakia origin developed 2 to 4 years after the onset of a white plaque, some occurred decades later, and only rarely did cancer occur quickly (Bouquot *et al.* 1988; Silverman *et al.* 1984). In another study, Pindborg *et al.* (1968) found that 36% of their subjects developed malignancy within 1 year and 64% after 1 year. Half of the 64% developed cancer after 3 or 4 years. Again the risk factor of duration has limited value in cancer prediction.

I.5.1.5. History of head and neck cancer

Individuals with a history of aerodigestive tract cancers have an increased risk (10%–30% higher) of a second primary cancer or local-regional recurrence (Grant *et al.* 1993). This poor prognosis is generally attributed to either undetected micro-invasion of primary tumours leading to an outgrowth of malignant cells after treatment and, eventually, recurrence at the former cancer site, or multi-focal carcinogenesis as a result of field cancerization. Because of this high cancer risk, patients with a history of head and neck cancer are followed up long-term.

Unfortunately this risk factor is also limited in its value. Patients with a history of oral cancer generally have a tendency to develop reactive lesions even to minor traumas that would not normally result in reactive changes because of the aggressive cancer

treatment that leaves the oral tissue fragile and hard to heal in response to trauma (Epstein and Scully 1997). Therefore, physicians tend to be reluctant to perform biopsies in these patients, and this frequently results in a delay of the diagnosis (Epstein and Scully 1997). Hence, any visual aid that can assist in detecting carcinomas and malignant margins would be in great demand in the medical field, as it would improve the survival rate and prognosis for oral cancer patients.

I.5.1.6. Other clinical risk factors for oral cancer

Age: The mean age at diagnosis of oral premalignancy is 50-69; less than 5% of diagnoses are in patients under 30 years of age (Kaugars *et al.* 1988; Lumerman *et al.* 1995; Mincer *et al.* 1972). Furthermore the aging process itself is the greatest risk factor for premalignant and malignant changes.

Habits and genetic predisposition: Heavy smoking and drinking are strongly associated with oral cancer in the Western world. In this sense, heavy smokers and drinkers, particularly those of older age, are at risk for oral cancer, although the majority of these people do not develop cancer. Ironically, OPLs found in people without apparent etiologies (non-smoker and social drinker) have higher cancer risk than those OPLs in heavy smokers and drinkers. It is generally presumed these people are genetically predisposed to oral cancer.

Gender: Although oral cancer is more common in males than in females (Silverman 1994; van der Waal *et al.* 1997), OPLs in females have higher risk than OPLs in males. The exact mechanism for this is unclear.

Candidiasis: Candidiasis is a fungal infection. Studies have suggested that oral leukoplakias with candidiasis are at a higher risk of developing into cancer than those without candida infection, thus raising the possibility that candidiasis of oral leukoplakia is a risk predictor (Krogh *et al.* 1987). However, it is not clear whether candidiasis is an independent risk factor or if it is only a reflector of non-homogeneous leukoplakia and dysplasia.

I.5.2. Histopathology - the current golden standard

Clinical risk factors help clinicians to decide whether a lesion needs to be biopsied and where to biopsy. The biopsy sample will then be sent for histopathological evaluation. Currently, histological diagnosis is the gold standard for determining the cancer risk for premalignant lesions. The gold standard or pathological risk factor is based mainly on the presence and degree of histological changes called "dysplasia." Dysplasia, as Lumerman describes, "is the diagnostic term used to describe the histopathological changes seen in a chronic, progressive, and premalignant disorder of the oral mucosa" (Lumerman et al. 1995). The criteria used to diagnose dysplasia are the following (WHO 1978):

Loss of polarity of the basal cell;

Presence of more than one layer of cells having a basaloid appearance;

Increased nuclear–cytoplasmic ratio;

Drop-shaped rete processes;

Irregular epithelial stratification;

Increased number of mitotic figures;

Presence of 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 prickle layers.

Lesions with dysplasia are further classified into mild, moderate and severe dysplasia. As shown in Figure 1, the degree of dysplasia is determined by the extent of spread of dysplastic cells in the epithelial layers. In mild dysplasia, the cytological and architectural changes are seen in the lower third of the epithelium; in moderate dysplasia, such changes are seen in the lower half of the epithelium; in severe dysplasia, the dysplasia involves the lower two third of the epithelial layers.

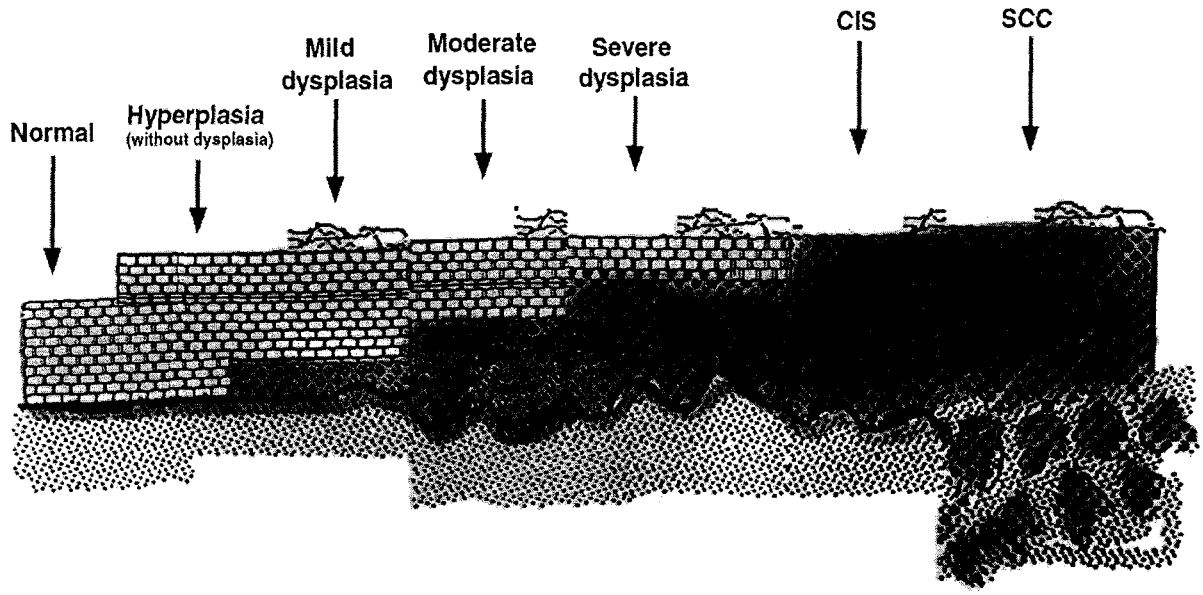

Figure 1 Histological progression model of oral cancer

The presence of dysplasia is a cancer risk predicting factor. Leukoplakia showing dysplasia is considered to have a higher risk for malignant transformation than those without dysplasia (Banoczy and Csiba 1976; Lumerman *et al.* 1995; Waldron and Shafer 1975). Epstein *et al.* (1996) reported that 43% of dysplastic leukoplakia progressed to malignancy. Another study conducted by Silverman *et al.* in 1984 found that 36% of leukoplakia with dysplasia became cancerous after 7.2 years, compared with only 15% of leukoplakia without dysplasia.

The degree of dysplasia is an important factor of cancer risk. The higher the degree of dysplasia an OPL has, the higher the cancer risk. Literature supporting this not only comes from studies of the oral cavity but also of other organs such as uterine cervix, lung, oesophagus, skin, pharynx, and larynx with well-accepted histological progression

model. As shown in Figure 1, oral SCC is believed to progress from hyperplasia to an increasing degree of dysplasia, to *CIS*, and finally to invasive carcinoma. *CIS*, a lesion regarded as cancer, is in fact closer to severe dysplasia in both pathology and clinical behaviour than to invasive SCC. Like severe dysplasia, changes of *CIS* are confined within the epithelium without destruction of the basement membrane. *CIS* generally has dysplastic changes involving the whole epithelial layers (bottom-to-top effects). However, a diagnosis of *CIS* can be made when the dysplasias only involve the lower two thirds of the epithelial layers when the cytological changes are pronounced. Both oral severe dysplasia and *CIS* have a high chance of progressing into invasive cancer, but neither have metastasis because of the lack of invasion. The diagnosis of invasive SCC is based on the invasion of dysplastic cells through the basement membrane, the destruction of underlying connective tissue, and growth of tumour islands in the connective tissue. The presence and degree of dysplasia are the gold standard in the diagnosis of cancer risk for many organs, and they have been time tested. The gold standard is excellent in guiding clinicians in the management of severe dysplasia and *CIS* since these lesions have a high chance of progression into invasive lesions.

However, even the gold standard has its limitations, since it is not good in predicting the cancer risk of those OPLs without dysplasia or with low-grade dysplasia. As mentioned previously, 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, new methods to identify the small percentage of progressing low-grade lesions from the majority of non-progressing lesions are highly desired.

A further limitation of dysplasia as a diagnostic criterion is that the identification of dysplasia needs a biopsy, while a biopsy needs the clinical presence of a lesion. Unfortunately, dysplasia per se is not visible clinically. It is only visible when it is accompanied by an increase in epithelial or keratin thickness or both (leukoplakia) or a decrease in epithelial thickness (erythroplakia). Visual aids that do not depend upon clinical appearance of leukoplakia to identify dysplasia are needed.

I.6. Critical genetic changes during oral cancer development

Over the last couple of decades, there has been an increasing emphasis on the use of molecular approaches, largely due to rapid advances in the area and easier application for both laboratory and clinic. Like the pathology which introduced the microscope to aid diagnosis, molecular biological methods boost diagnosis to a molecular level which can yield a much higher resolution than microscopy. The significance of introducing molecular methods lies in two aspects. First, they will facilitate the understanding of the mechanisms of early carcinogenesis. Second, they will directly impact on the clinical management of these lesions.

I.6.1. Molecular markers of cancer development

The traditional histopathological marker of grading of oral epithelial dysplasia is still extensively used, but its prognostic value is very limited mainly because of its poor predictive value for low-grade dysplasia and poor reproducibility (Karabulut *et al.* 1995). Oral cancer, at the molecular level, represents an accumulation of molecular lesions that control cell cycle, cell survival, cell motility, and angiogenesis (Regezi and Jordan 2001). With molecular biology technique advances in recent years, considerable progress has

been made in understanding the genetic basis of the development of oral cancer. Moreover, molecular markers, which are linked to malignant transformation, may provide a non-invasive detection and non-surgical therapeutic approach by targeting these molecules.

It is well accepted that cancer is the result of an accumulated, stepwise progression of genetic alterations which produces a clonal population of cells (Fearon and Vogelstein 1990). Although the precise order and number of events required for carcinogenesis remains unknown, a recent series of studies demonstrated six important steps that are believed to be necessary for a cancer to develop (Hahn *et al.* 1999; Hanahan and Weinberg 2000). These steps include the following: 1) Acquisition of autonomous proliferative signalling; 2) Inhibition of growth inhibitory signals; 3) Evasion of programmed cell death; 4) Immortalization; 5) Acquisition of a nutrient blood supply (angiogenesis); and 6) Acquisition of the ability to invade tissue (Das and Nagpal 2002).

There are two major types of genes, oncogenes and tumour suppressor genes (TSGs), which are critical to carcinogenesis. Both of them will be discussed in the following section.

I.6.2. Oncogenes and tumour suppressor genes

I.6.2.1. Oncogenes

A proto-oncogene is a gene that is involved in signal transduction and execution of mitogenic signals, usually through its protein product. Upon activation, it or its product becomes a tumour inducing agent, an oncogene.

Under normal circumstances, proto-oncogenes encode proteins that positively regulate critical cell growth functions, such as proliferation, cell growth, cell motility, internal cell signalling and angiogenesis (Regezi and Jordan 2001). They include kinases, transcription factors, regulators of cell cycle or growth factor receptors and their ligands.

Activation of proto-oncogenes to oncogenes can be due to many causes including xenobiotics, such as dietary carcinogens, low intake of free radical scavengers and antioxidants, ionizing and ultraviolet radiation, and DNA replicating errors during meiosis or mitosis and viruses. Proto-oncogenes may be rendered in four oncogenic ways—deregulation of expression, gene mutation, translocation and amplification (Regezi and Jordan 2001).

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

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

I.6.2.2. Tumour Suppressor genes (TSGs)

A tumour suppressor gene produces a protein that is a negative regulator of many cellular activities. As such, it often acts to reduce the probability that a cell will turn into a tumour cell. As implied by the name, suppressor genes counterbalance the growth and mitotic potential of proto-oncogenes. Usually they are believed to be recessive genes that negatively regulate cell proliferation and promote apoptosis and differentiation. TSGs are biochemical diverse including phosphatases, kinases, cyclin-dependant kinase inhibitors, transcription factors, cell adhesion molecules, protein degradation proteins and DNA repair molecules (Jones 2004).

Alterations of the genes in this group essentially accelerate proliferation of a clone of cells. To have a phenotypic effect, differences in or loss of both maternal and paternal gene copies (alleles) are required. According to the Knudson 2-hit hypothesis, alteration of one allele followed by alteration of the other allele leads to completely loss of its function. Thus, inactivation of both alleles confers the tumour phenotype (Michael *et al.* 2005; Regezi and Jordan 2001). If only one copy is absent, then it will put such a patient

at a risk of developing a malignancy but the actual alteration to cell behaviour will occur until the second allele is lost (Jones 2004).

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

I.6.3. LOH and microsatellite analysis

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

I.6.3.1. Loss of heterozygosity (LOH)

Usually every eukaryotic gene has two alleles for each gene. One is maternal while the other is paternal. LOH in a cell represents the loss of a single parent's contribution to part of its genome. LOH can arise from many mechanisms including

chromosome loss, mitotic recombination, gene or chromosomal region deletion, or other events result in alteration of copy number.

The concept of LOH in cancer is consistent with Knudson's two-hit hypothesis, which states that inactivation of one of the two alleles of tumour suppressor genes by either a germ line or somatic mutation is a critical step in carcinogenesis because only one more mutation inactivation is required to the remaining allele before expression of the phenotype occurs (Knudson 1985). The typical example is hereditary retinoblastoma, in which a single parent's contribution of the tumour suppressor Rb1 is flawed in every cell of the patient's body. Although most cells will have a functional second copy, chance of losing the other allele in individual cells almost inevitably leads to the development of this retinal cancer in the young child.

LOH can be identified by noting the presence of heterozygosity at a genetic locus in an organism's genome, and the absence of heterozygosity at that locus in the cancer cells. LOH is often determined using polymorphic markers, such as microsatellite or single nucleotide polymorphisms (SNPs), for which the two parents contributed different alleles. LOH analysis has been used in the discovery of some important genes, including *APC* (adenomatous polyposis coli) gene, *DCC* gene, *DPC4* gene and *ATM* gene, etc.

I.6.3.2. Methods to detect LOH

Currently three methods are available for the study of LOH: restriction fragment length polymorphism (RFLP) analysis, microsatellite analysis, and SNPs analysis.

RFLP

This is a key tool in DNA fingerprinting, reflecting the existence of different alleles in the individual. DNA are amplified (typically by PCR) and cut with certain restriction enzymes to look for the variation in the length of DNA fragments, called polymorphism. Such polymorphisms are generated by mutations that create or abolish recognition sites for these enzymes. If a polymorphism can be identified close to the locus of a genetic defect, it provides a valuable marker for tracing the inheritance of the defect.

Microsatellite analysis

A microsatellite is a short, DNA sequence (a tandem repeat of DNA sequence) that is repeated many times within the genome of an organism. Many repeats tend to be concentrated at the same locus.

In a microsatellite, the repeated sequence is very simple, consisting of two to four nucleotides, and can be repeated 10 to 100 times. Classically, the first microsatellite identified were dinucleotide repeats often called "Ka-Ka" repeats or "CACACA. . .". These are highly frequent in human and other genomes, and present every few thousand base pairs. Variability or dynamism of a dinucleotide repeat is typically a function of size: repeats larger than 10-15 repeats and without interruptions tend to be polymorphic. Larger repeats often are more polymorphic.

The number of repeats at a particular locus is highly polymorphic between individuals of the same species. It is for this reason that microsatellite sequences can be used for genetic fingerprinting and paternity testing. Most loci of the genome, even non-coding parts, would be too similar to allow individuals to be reliably distinguished. They

are often less useful in evolutionary studies since they are unstable and prone to mutating to different sizes.

The hyper-variability arises because the repeated simple sequences cause a high frequency of loss or insertion of additional repeats by confusing the DNA replication machinery. Self-complementary sequences may aid this process.

In tumour cells, where controls on replication may be damaged, microsatellites may be gained or lost at an especially high frequency during each round of mitosis. Hence a tumour cell line might show a different genetic fingerprint from that of the host tissue.

The approach involves the examination of DNA for microsatellites within the chromosomal DNA. A polymerase chain reaction (PCR) is performed on the microsatellite areas comparing DNA from lesion from DNA from normal tissue. The amplified material is then run through electrophoresis. By checking the signal intensity of the two bands with normal control DNA in tumour or lesion samples, we can determine if LOH present.

The basic rationale for use of this assay is that a frequent finding of an allele imbalance in a particular segment of a chromosome in a tumour type is highly suggestive of the loss of a critical TSG or amplification of an oncogene within this region. Loss of heterozygosity usually suggests that a putative TSG nearby also may be lost. The detection of allele imbalance in the clinical sample demonstrates the presence of a clonal population of cells that share altered genetic information, a characteristic of cancer cells (Cairns and Sidransky 1999).

The advantages of using microsatellite markers in an LOH assay are many fold. First, microsatellite markers are highly polymorphic and well distributed throughout the human genome. They show levels of heterozygosity between 30-80%, significantly above the level observed with RFLP analysis, which is based on base substitutions at endonuclease recognition sites. Second, this γ^{-32}P end-labelled PCR-based approach is much more sensitive than RFLP analysis, requiring only a small amount of DNA (4 ng per reaction), which is critical for the study of premalignant lesions. Another advantage is that microsatellite markers can be used on DNA from paraffin-embedded archival samples, in addition to fresh or frozen samples, which is critical for a retrospective study of samples. For these reasons, this thesis employs microsatellite analysis.

SNPs array

SNPs are the most common form of sequence variation in the human genome. They occur approximately in every 1, 200 bp and at more than 2 million sites in the genome, so it's possible to place SNPs at high density along the genome (Marth *et al.* 2001; Sachidanandam *et al.* 2001).

SNP arrays have been introduced for genome-wide screening of chromosome imbalance and are becoming more widely used. This high-throughput polymorphism detection technology holds great promise for the characterization of complex diseases including cancer (Zheng *et al.* 2005). High-density mapping of genetic losses reveals potential tumour suppressor loci and will be useful in the clinical classification of individual tumours. SNP arrays can be applied effectively to detect small regions of chromosomal changes and provide more information regarding the boundaries of loss

regions. Furthermore, more markers increase confidence in a detected event (van der Waal *et al.* 2000).

SNP arrays have successfully been employed in loss-of-heterozygosity (LOH) identification (Rauch *et al.* 2004; Wang *et al.* 2004; Zheng *et al.* 2005). The newly developed high-density SNPs array, which combines the power of microsatellite analysis and Comparative Genomic Hybridization, can provide a precise and high-resolution mapping of genetic alterations (Zhou *et al.* 2005). Once allele-specific copy number is determined, we will not only be able to detect the allele imbalance but also can infer that the imbalance is due either to the amplification of one of the alleles or to the deletion of the other.

SNP array assay is accurate, automatic, and readily adaptable to the clinical setting and high-density mapping (Zheng *et al.* 2005). Allelotyping with SNP array can save considerable time over microsatellite analysis. However, the procedure still takes a considerate amount of DNA even with multiplex amplification before hybridization, and requires good quality DNA. Furthermore, the approach is costly. Microsatellite analysis of key regions associated with progressing to cancer is a cost effective, DNA sparing approach. It can yield robust results even with DNA from archival samples which are often of low quality, especially with older specimens.

I.6.3.3. LOH at critical chromosome regions in oral cancer and HNSCC

Many studies have shown that the loss of one or several specific regions of chromosomes that harbour tumour suppressor genes is a common event in oral malignancies (Ah-See *et al.* 1994; Nawroz *et al.* 1994; Rosin *et al.* 2000). However,

there are only a few studies on genetic changes in OPLs that may further progress into oral SCC.

In this thesis, microsatellite markers on chromosome 3p, 4q, 8p, 9p, 11q, 13q, and 17p will be used to evaluate the risk OPLs, as they have been previously reported to be potential risk indicators associated with progression of OPLs (Rosin *et al.* 2000). Each of these regions will be discussed as follow. Table 1, at the end of the summary, provides frequency data for each arm for HNSCC and oral cancers in the literature.

Chromosome 3

Allelic loss of 3p is predicted as an early and basic event in HNSCC development (Bockmuhl *et al.* 1996). Numerous studies have shown frequent LOH at the 3p region, with an average deletion ratio of 52%. Based on discrete regions of deletion on the 3p arm in a variety of human cancers, several candidate TSGs have been suggested, including *VHL, FHIT, RASSF1*, and *H37* (Dammann *et al.* 2000; Kisielewski *et al.* 1998; Latif *et al.* 1993; Oh *et al.* 2002; Tran *et al.* 2005). Finemapping suggests several regions of interest, two of which are more frequently reported at 3p14 and 3p21 (Maestro *et al.* 1993; Partridge *et al.* 1996). Each region is presumed to harbour one or more putative TSGs.

Within the region at 3p14.2 is one of the most common fragile sites in the genome, called *FRA3B*. Fragile sites are areas that are extremely weak and break easily in chromosomes that may be targeted by carcinogens. The gene *FHIT* was identified in 1996 in this region and proposed as a TSG for many human cancers (Gonzalez Baron

1998; Huebner and Croce 2003; Mao *et al.* 1996; Mao *et al.* 1998; Pennisi 1996; Sozzi *et al.* 1996; Wilke *et al.* 1996; Wu *et al.* 1994). The *FHIT* locus frequently exhibits deletions in preneoplasias and majority cancers (Huebner and Croce 2001). The gene is widely studied with more than 350 recent reports. An association of gene mutation has been reported with high proliferative and low apoptosis indices, node positivity, loss of mismatch repair protein, likelihood of progression and reduced survival (Huebner and Croce 2003).

FHIT encodes a protein with 69% similarity to a *Schizosaccharomyces pombe* enzyme, diadenosine 5', 5"'-P1, P4-tetraphosphate (Ap4A) asymmetrical hydrolase which cleaves the AP4A substrate into 5' - ADP and AMP. Current theories suggest that diadenosine tetraphosphate may accumulate in cells in the absence of the normal expression of the gene and may eventually lead to dysregulated DNA synthesis and cell replication (Mao *et al.* 1996).

TSGs that are responsible for LOH at the other two other regions (3p24-pter, and 3p21. 3) are still not identified. For example, the 3p24-25 region contains the *VHL* gene, which is thought to be a member of a novel class of glycan-anchored membrane proteins that function in signal transduction and cell adhesion (Waber *et al.* 1996). Its alteration has been reported in cancers, especially in those that are *VHL*-associated (Decker *et al.* 1997; Kok *et al.* 1997; van den Berg *et al.* 1997). Uzawa mentioned the possibility that the *VHL* gene may in involved in oral SCC development (Uzawa *et al.* 1998). However, mutations of the *VHL* gene could not be identified and the examination of this gene for other methods of inactivation, such as by hypermethylation, has yielded negative results. It is possible that allelic loss of chromosome arm 3p in HNSCC involves regions

surrounding the *VHL* locus but not the *VHL* gene itself. Another TSG in HNSCC may exist in the regions surrounding D3S1110 at 3p25 (Uzawa *et al.* 1998; Waber *et al.* 1996).

Chromosome 4

Deletions on chromosome 4 were suggested to be associated with the late stages of HNSCC (Califano *et al.* 1996). Our study followed two different loci on this chromosome: 4q24~25 and 4p15. The former region has been reported as a frequently LOH in HNSCC in several publications (Pershouse *et al.* 1997; Wang *et al.* 1999).

The locus, 4p15, has been found to be significantly deleted in breast carcinoma, small cell lung carcinoma, and colorectal carcinoma in addition to reports of alteration in oral cancer (Girard *et al.* 2000; Shivapurkar *et al.* 2001). Despite these regions showing a high incidence of deletion, none of the genes located in these regions have been shown to fulfil the requirements for a TSG.

Chromosome 8

Region 8p23 was found with a high frequency of LOH in HNSCCs (Sunwoo *et al.* 1999; Wu *et al.* 1997). In our studies we have reported the association of LOH at markers *D8S262* and *D8S264* with increased risk of progression. LOH is present in 11/21 (52%) progressing low-grade dysplasia compared to 8/51 (15%) of non progressing lesions of same histology ($P = 0.003$) (Rosin *et al* 2000.).

LOH has been also reported for 8q, at a high frequency at 8q13 and 8q24 in HNSCCs (Okamoto *et al.* 2003). In other solid tumours, the 8q24 loci in hepatocellular

carcinoma and lung cancer (Girard *et al.* 2000; Li *et al.* 2001) and the 8q12-13 region in prostate cancer (Perinchery *et al.* 1999) were found to be commonly deleted. Conversely, the chromosomal region of 8q24 was also found to be amplified in various malignancies, including hepatocellular carcinoma (Wang *et al.* 2001), prostate cancer (El Gedaily *et al.* 2001), and endometrial carcinoma (Suehiro *et al.* 2000). To date there is no information on progression risk for OPLs for any of these regions on 8q.

Chromosome 9

LOH on 9p is by far the most commonly reported chromosomal defect in head and neck cancers, with LOH reported in the majority of malignant lesions. This region contains at least four TSGs (*p15, p16, p19*, and *MTAP*) are located (Field *et al.* 1995). On the 9q arm, frequent LOH at the 9q31.3 and 9q34.2 markers have been reported in HNSCC, but at lower frequencies (Ah-See *et al.* 1994; Field *et al.* 1995; Okamoto *et al.* 2003).

The majority of attention from the standpoint of gene identification has focused on the cell cycle gene *p16* (also know as *MTS-1* for major tumour suppressor-1, *INK4a* for inhibitor of cyclin-dependent kinase 4a, and *CDKN2A* for cyclin-dependent kinase inhibitor 2A). *p16* (*INK4A/MTS-1/CDKN2A*) encodes a cell cycle protein that inhibits cyclin-dependent kinases (CDK) 4 and 6, preventing phosphorylation of Rb protein and consequently inhibiting the cell cycle transition of the G1-S phase (Reed *et al.* 1996). The major biochemical effect of *p16* is to halt cell-cycle progression at the G1/S boundary. Approximately 80% of the head and neck cancers and premalignant lesions have inactivated *p16* at the protein and/or DNA level, suggesting that inactivation of *p16*

may play an important role in early head and neck cancer development (Papadimitrakopoulou *et al.* 1997; Reed *et al.* 1996).

Mutations of this gene are not apparently frequent in oral cancer (Dawson *et al.* 1996; Reed *et al.*). The gene is inactivated by alternative mechanisms including homozygous deletion and methylation of the 5'CpG-rich region, which results in a complete block of gene transcription (Matsuda *et al.* 1996; Merlo *et al.* 1995; Papadimitrakopoulou *et al.* 1997; Rawnsley *et al.* 1997). Of interest, the same gene codes for another protein ARF created by alterative splicing of the *p16* gene. ARF binds to and promotes the degradation of ubiquitous ligase, MDM2, which targets p53. This leads to the stabilization and accumulation in the cell of p53 allowing it to perform its critical function in triggering cell cycle arrest and cell death in damaged cells (Finch *et al.* 2006; Lindstrom and Zhang 2006; McGurk *et al.* 2005; Sherr 2006).

Chromosome 11

A region at 11p13~14 has shown high LOH frequencies in HNSCC and oral cancer (Bockmuhl *et al.* 1996). This region contains the *cyclin D1* gene, another gene that plays a central role in cell proliferation. This region is frequently lost in other tumor types also, such as oropharyngeal carcinoma (Harn *et al.* 2002; Hermsen *et al.* 1996).

Chromosome 13

Multiple minimal deleted regions including 13q14 and 13q33-34 were determined for 13q in HNSCC occurring as frequent event in HNSCC (Beder *et al.* 2003; Maestro *et al.* 1996; Sunwoo *et al.* 1999). The *ING1* gene may be a TSG that is located in the 13q33~34 region of loss (Gunduz *et al.* 2000). The 13q14 region contains the *Rb* gene.

However it is not commonly mutated in oral cancers. At least two candidate TSGs have been identified and proposed as TSGs in diverse tumor types including HNSCC (Maestro *et al.* 1996; Sunwoo *et al.* 1999).

Chromosome 17

Deletion of the chromosome 17p13 region, which harbours a well-known TSG, *p53*, has been reported to be a frequent occurrence in the transition from the pre-invasive to the invasive lesion in various tumour types, including HNSCC (Boyle *et al.* 1993). The 17p13 is the most frequent region of alteration on this arm (Beder *et al.* 2003).

Table 1 LOH frequencies in head and neck or oral cancers

Chromosome Arm	Oral Cancer	Head and Neck Cancers
3p	14/27[1] Wu *et al,* 1994 17/21 Partridge *et al,* 1994 26/45 Ishwad *et al,* 1996 34/48 Partridge *et al,* 1996 15/25 Grati *et al,* 2000 41/78 Jang *et al,* 2001 26/36 Kayahara *et al,* 2001 22/35 Rosin *et al,* 2002 32/39 Zhang *et al,* 2002 24/40 Arai *et al,* 2002 14/40 Yamamoto *et al,* 2003 **32-82%**	28/38 Maestro *et al,* 1993 8/18 Ah-See *et al,* 1994 32/61 Adamson *et al,* 1994 9/19 el-Naggar *et al,* 1995 18/27 Califano *et al,* 1996 18/27 Nawroz *et al,* 1994 22/46 Rowley *et al,* 1996 25/50 Gonxalez *et al,* 1998 165/302 Shiga *et al,* 2004 **44 - 74%**
4q	13/39 Zhang *et al,* 2002 18/40 Shinno *et al,* 2005 19/20 Lin *et al,* 2005 **33-95%**	20/27 Pershouse *et al,* 1997 **75%**

Chromosome Arm	Oral Cancer	Head and Neck Cancers
8p	21/35 Wu *et al,* 1997 23/29 Ishwad *et al,* 1999 17/25 Grati *et al,* 2000 20/39 Zhang *et al,* 2002 18/27 Zhou *et al,* 2005 **51-79%**	11/36 Li *et al,* 1994 8/20 Nawroz *et al,* 1994 14/40 Field *et al,* 1995 10/19 El-Naggar *et al,* 1995 8/20 Califano *et al,* 1996 13/44 Scholnick *et al* 1996 23/51 Ishwad *et al,* 1999 85/150 Coon *et al,* 2004 **31 - 57%**
9p	35/73 Ishwad *et al,* 1996 19/25 Grati *et al,* 2000 15/39 Partridge *et al,* 2000 60/96 Jang *et al,* 2001 10/24 Xiao *et al,* 2001 28/36 Rosin *et al,* 2002 29/39 Zhang *et al,* 2002 16/27 Tran *et al,* 2005 17/27 Zhou *et al,* 2005 **48-74%**	21/29 van der Riet *et al,* 1994 21/29 Nawroz *et al,* 1994 24/39 Field *et al,* 1995 13/18 El-Naggar *et al,* 1995 54/74 Califano *et al,* 1996 17/42 Lydiatt *et al,* 1997 21/43 Partridge *et al,* 1999 172/302 Shiga *et al,* 2004 75/150 Coon *et al,* 2004 41/42 Xu *et al,* 2004 **45 - 98%**
11q	14/25 Uzawak *et al,* 1996 19/39 Zhang *et al,* 2002 17/27 Zhou *et al,* 2005 **49-63%**	14/23 Nawroz *et al,* 1994 9/20 Ah-See *et al,* 1994 3/39 Field *et al,* 1995 5/15 El-Naggar *et al,* 1995 13/52 Lazar *et al,* 1998 9/23 Venugoaplan *et al,* 1998 21/34 Tan *et al,* 2004 **23-62%**
13q	23/34 Ogawarak *et al,* 1998 4/32 Rosin *et al,* 2002 16/39 Zhang *et al,* 2002 **13-68%**	12/22 Nawroz *et al,* 1994 16/60 Li *et al,* 1994 31/60 Yoo *et al,* 1994 8/30 Field *et al,* 1995 31/60 Califano *et al,* 1996 32/48 Maestro *et al,* 1996 **27-67%**

Chromosome Arm	Oral Cancer	Head and Neck Cancers
17p	12/39 Partridge *et al,* 2000 32/54 Jang *et al,* 2001 13/36 Rosin *et al,* 2002 72/172 Zhou *et al,* 2005 **31-42%**	6/19 Ah-See *et al,* 1994 19/38 Adamson *et al,* 1994 9/20 Li *et al,* 1994 12/22 Nawroz *et al,* 1994 18/36 Field *et al,* 1995 3/14 El-Naggar *et al,* 1995 34/62 Califano *et al,* 1996 12/33 Partridge *et al,* 1999 24/54 Schneider *et al,* 2004 172/302 Shiga *et al,* 2004 **21-57%**

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

I.6.4. Molecular progression model for oral cancer development

Early in 1976, Nowell suggested that neoplastic transformation occurred from a single stem cell that had a critical genetic alteration that gave it a growth advantage over its neighbor cells. This mutation resulted in an outgrowth of a clone of altered cells (initiation), in which further mutation occurred by exposure to carcinogen (promotion). Over time, with the accumulation of more mutation and repeated clonal outgrowths alterations became apparent in epithelial cell behavior and structure (progression), generating the clinical and microscopic alterations to histology that define premalignant stages and invasive lesions.

In 1990 Fearon and Vogelstein proposed the first molecular progression model for colorectal cancer (Fearon and Vogelstein 1990). This tumour progression model extends from 3 basic principles: i) Neoplasms are the results of activation of proto-oncogenes and inactivation of TSGs; ii) There exists a defined order of genetic events leading to the

development of a tumour phenotype; and iii) The net accumulation of genetic alterations rather than their order determines the malignant phenotype (Vieth *et al.* 2004). Molecular progression models were proposed in other tissues and organs in the following years.

In 1996 allelic loss was investigated in the whole spectrum of premalignant and malignant lesions of head and neck cancer, including hyperplasia, dysplasia, *CIS*, and SCC and suggested a molecular progression model for HNSCC (Califano *et al.* 1996). The model proposed that LOH at 9p is the earliest event associated with transition from normal to benign hyperplasia; LOH at 3p and 17p is associated with dysplasia. *CIS* and SCC were characterized by additional deletions on 4q, 6p, 8, 11q, 13q, and 14q. However, the study merged all dysplasias (mild, moderate and severe) into one study group. While the majority of mild dysplasia will not progress into cancer, severe dysplasia, similar to *CIS*, has a much higher probability of cancer progression. Therefore, subdivision of molecular profiles at different stage of dysplasia will enable us to better understand the progression pathway of oral cancer.

In 2001, Rosin *et al.*, this research team, further refined this molecular progression model for oral SCC by investigating all degrees of oral dysplasias by means of LOH analysis using multiple microsatellite primers for the seven chromosomal regions (3p, 4q, 8p, 9p, 11q, 13q, and 17p) known to be frequently lost in oral tumours. The data in this study supported the findings from previous studies that accumulations of genetic changes are critical for tumour progression, and further proposed that specific patterns of allelic loss occurred at different degrees of dysplasia.

I.6.5. LOH patterns predict cancer risk of primary OPLs

Studies have shown that OPLs with LOH at 9p21 and/or 3p14 have increased risk of malignant transformation. Mao's group found that 37% of OPLs with LOH progressed into cancer compared to only 6% of lesions without LOH (Mao *et al.* 1996). Another study showed that 94% of hyperplasia and dysplasia lesions with 9p and/or 3p losses progressed into SCC (Partridge *et al.* 1996).

In a paper from our research team, we examined LOH at 3p, 4q, 8p, 9p, 11q, 13q, and 17p in hyperplasia, mild, and moderate dysplasias in patients with or without progression into cancer. The progressing group acquired more LOH than non-progressing group. 97% of the cases in this group exhibited loss at 9p and/or 3p, with additional losses noted at higher frequency on all other arms. Rosin *et al.* proposed that individuals with premalignant lesions can be placed into 3 categories of risk depending on LOH patterns: 1) low risk: retention at 3p and 9p; 2) intermediate risk: loss at 3p or 9p; and 3) high risk: loss at 3p and/or 9p plus other arms. The highest risk group had a 33-fold increase in cancer risk compared to the low-risk group. The study also suggested that 3p and/or 9p LOH is necessary for a premalignant lesion to progress and for malignant transformation to occur, further additional loss must be present on other arms (Rosin *et al.* 2000).

I.6.6. LOH patterns predict cancer risk of second oral malignancy (SOM)

A recent retrospective study from this lab has investigated the utility of LOH as a marker to identify OPLs in former cancer patients that were second oral malignancies at high risk for progression into SOM (Rosin *et al.* 2002). The LOH data on chromosomes

3p, 4q, 8p, 9p, 11q, 13q and 17p in leukoplakia lesions at former cancer sites with known outcome showed that 3p and /or 9p loss in these post-treatment leukoplakias was associated with a 26.3-fold increase in the risk of developing SOM compared with those that retained both of these arms. The significant LOH information collected in this study represents a possible use of 3p and 9p loss in post-treatment lesions as a simple and direct test for stratifying risk of SOM development. The data strongly suggest that the identification of such alterations at a former cancer site should alert the clinician to the presence of a potentially aggressive lesion, even if the histological diagnosis is hyperplasia or mild dysplasia, and even if distinction between SPT and recurrence could not be determined.

These studies suggest that LOH could be a molecular marker for cancer risk prediction since it can distinguish OPLs with high progression risk from those with low progression risk in the clinically and histologically similar lesions.

I.7. The need for visual aid clinical examination

Unfortunately, both the gold standard in pathology and molecular analyses suffer from a common limitation: they rely on the clinician's visual inspection for a decision of whether or not to biopsy a lesion, and on his or her ability to correctly localize the biopsy to the area of the lesion with the greatest risk of progression. However, benign lesions may present very similar clinical appearance to premalignant lesions, so localization of biopsies is very difficult based on clinical appearance even for the experienced clinician. Furthermore, some high-risk lesions are not clinically apparent or visible. Thus, a visual aid that can facilitate the selection of high cancer risk sites and biopsy sites is badly

needed. This kind of visual aid should meet all the following requirements (De Veld *et al.* 2005):

1) A technique that can distinguish between different lesion types in a reliable and non-invasive way would be very useful.

2) A device providing *in vivo* lesion classification would be particularly useful for finding the optimal biopsy site, so that the risk of under-diagnosis and need for repeated biopsies is avoided.

3) The device should detect premalignant changes and lesions in an earlier stage, preferably before visual detection is possible.

Autofluorescence imaging is an emerging aid that could facilitate the detection and classification of lesions. In this thesis, I will give an overview of the literature on autofluorescence imaging first and provide our data later.

I.8. Fluorescence visualization (FV)

I.8.1. Optical principles

While white-light visualization (visualization with naked eyes) can perceive only a fraction of the spectral characteristics that differentiate diseased tissue from its normal counterpart, optical methods, particular those based on fluorescence imaging and spectroscopy, will likely improve our ability to detect tissue changes, such as OPLs and early cancer. This section reviews optical principles before we discuss the fluorescent visualization.

Initially light was believed either to behave as waves or to be corpuscular particles in nature. After much debate and research, it has been concluded that there are circumstances in which light behaves like waves and those in which it behaves as particles (John Enderle *et al*, 2000). The characteristics of visible light are described by using a dualistic theory that combines wave theory and quantum theory, since light is a form of electromagnetic radiation (EMR). EMR also involves the movement of energy through space as a combination of electric and magnetic fields (de Paula *et al.* 1995). Other types of EMR are X-rays, ultraviolet (UV) rays, infrared radiation, microwaves and radio waves.

The wave theory of light maintains that light is propagated in the form of waves that travel at the velocity of light, 3×10^8 m/s in a vacuum, and exhibit in the properties of wavelength (λ), frequency (ν) and intensity (I). λ and ν are governed by the equation $\lambda \times \nu = 3 \times 10^8$ m/s. Wave theory accurately accounts for the reflection, refraction, and scattering properties of tissue optics.

Quantum theory depicts light as tiny, finite bundles of energy called photons. Each photon travels at the speed of light in a vacuum and contains a specific amount of energy. The relationship between wavelength and photon energy is described by the equation $E = h \cdot c / \lambda$, in which E is the energy, h is Planck's constant (6.626×10^{-34} J·s), and c is the speed of light in vacuum. Based on this equation, the shorter the wavelength, the more energy the photons will contain. Quantum theory is suited for describing the fluorescence properties of tissue optics.

Figure 2 is a schema showing the spectrum of EMR. As the frequency increases from the bottom to the top there is a decrease in wavelength for the different types of EMR, energy also accompanied by an increase in energy. Thus, within visible light, red light has a relatively longer wavelength than blue/violet. Infrared, which is invisible to humans, has an even longer wavelength than red. On the other side of the visible light spectrum, ultraviolet light has an even shorter wavelength than (visible) violet with a higher energy level. Excitation photons used in thesis include wavelengths from 400 to 460 nm (violet to blue).

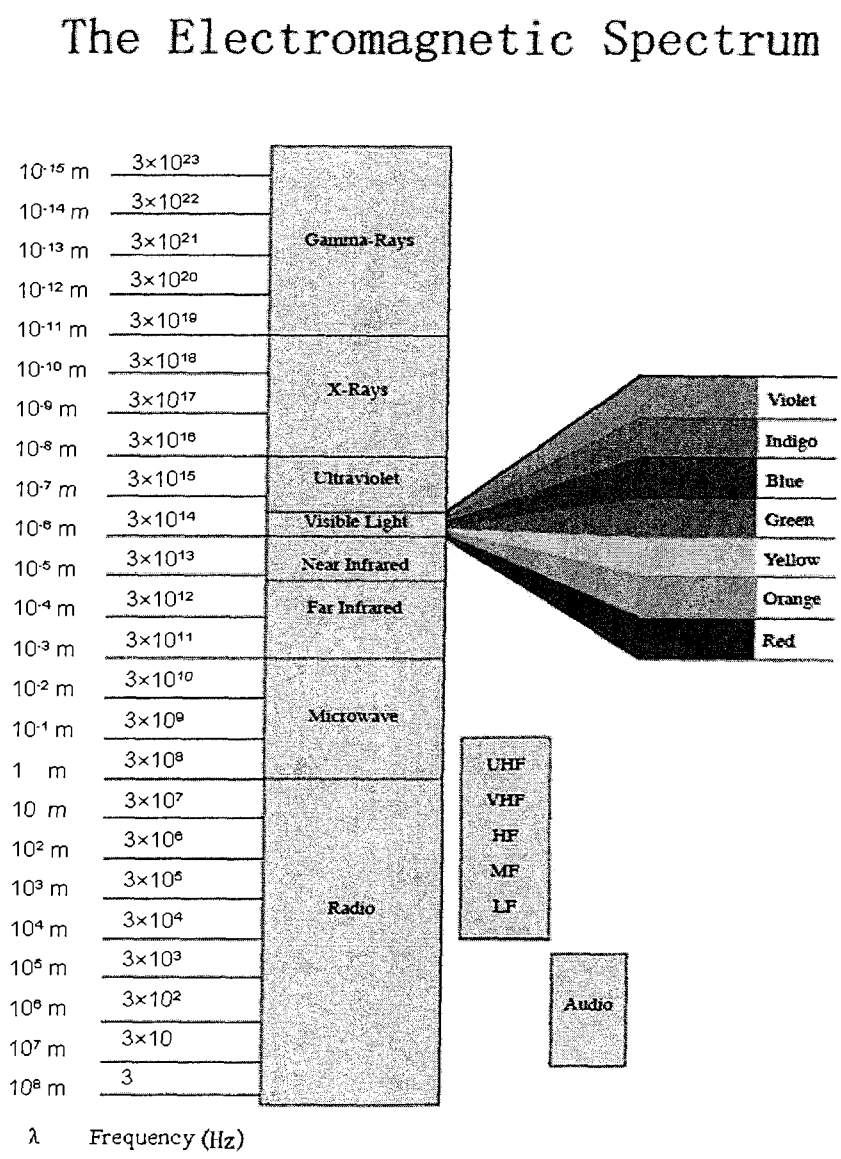

Figure 2 The electromagnetic spectrum.

This figure shows wavelengths for different types of electromagnetic radiation with an emphasis on visible light. (Adapted from http://lasp.colourado.edu/cassini/education/Electromagnetic%20Spectrum.htm , University of Colorado-LASP)

I.8.2. Tissue Optical Properties

It is believed that optical properties of epidermal tissue reflect its structure and chemical composition (Richards-Kortum and Sevick-Muraca 1996). When a beam of light reaches the skin surface, a part of it will be reflected by the surface, while the rest will be refracted and transmitted into the skin. The light transmitted into the epidermal tissue will then be scattered, absorbed and reflected at the boundaries.

After multiple scattering, some of the transmitted light will re-emerge through the tissue surface. This phenomenon is called diffuse reflection. The intensity of diffuse reflection is determined by both the scattering and absorption properties of the tissue. The stronger the absorption, the less the diffuse reflection will be. Conversely, the stronger the scattering, the more the diffuse reflection showed.

To date, several methods have been developed to measure tissue optical properties. The collimated transmission technique can be measured by the total attenuation coefficient ($\mu_a + \mu_s$). A beam of collimated light illuminates a thin piece of tissue. Un-scattered transmitted light is detected while the scattered light is rejected by use of a small aperture. The un-scattered transmitted light can be calculated by Beer-Lambert law with the following equation.

$$I(z)=I_0 \bullet \exp[-(\mu_a+\mu_s) \bullet z]$$

I(z) is the un-scattered transmitted light intensity after penetrating to a depth of z. In collimated transmission measurements, I_0, I(z)and z are measured. Therefore, the total interaction coefficient ($\mu_a + \mu_s$) can be deduced.

I.8.3. Principle of fluorescence

The electronic configuration of a molecule is usually known as its ground state. Upon absorption of a photon, an electron can be promoted from its ground state to an excited state through a process called electronic transition by the light energy. Then the electrically excited molecule rapidly returns to a more stable energy state by emitting a photon with less energy. The light that emits from the molecules will produce an emission light spectrum (Richards-Kortum and Sevick-Muraca 1996). The molecule with this ability is called a fluorophore. Since native fluorophores inside the tissue are responsible for this fluorescence, it is termed autofluorescence, endogenous fluorescence or natural fluorescence.

The amount of visible autofluorescence is determined by two factors:

(1). The light-producing component is positively associated with visible autofluorescence. When a tissue has an increased amount of fluorophores (quantity) or fluorophores with a higher emanation of fluorescence (quality), the tissue will show an increased amount of autofluorescence. The quantity and quality of fluorophores are determined by the biochemical properties of the target tissue, so changes of the biochemical properties of tissue would result in changes in autofluorescence.

(2) The light-blocking component is negatively associated with visible autofluorescence. When a tissue has an increased capacity of light scattering or reflecting and light absorbing, the tissue will show a decreased visible autofluorescence. The blocking components are associated with both the biochemical component of the tissue, and the structure and morphology of the tissue, such as histology of the epithelium.

I.8.4. Light-producing component (fluorophores) in oral tissue

There are numerous fluorophores in the human stratified squamous epithelium and the underlying stroma. There are three major fluorophores that are of importance to this study (Svistun *et al.* 2004):

1) Metabolic co-factors: nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD+);

2) Collagen and elastic fibre cross-links;

3) Porphyrin.

With excitation in the UV (300–400 nm) and near-UV (400–460 nm) ranges, emission of fluorescence from chromophores, such as NAD(*P*)H, flavins, and porphyrins are in 450-700 nm (blue to red light).

I.8.4.1. NADH and FAD^+

NADH and FAD^+ are water soluble cofactors that undergo reversible oxidation and reduction in many of the electron transfer reactions of metabolism. Indeed, NADH and FAD^+ fluorescence signals may be closely related to the cellular metabolic activity (Juan Manuel Benavides 2003; Pavlova *et al.* 2003).

Figure 3 shows the chemical structure of nicotinamide adenine dinucleotide (NADH). NADH is a coenzyme for oxidation reactions (de Paula *et al.* 1995). It is composed of two nucleotides joined through their phosphate groups by a phosphoric acid anhydride bond. One of nucleotides is linked with nicotinamide (derived from the

vitamin nicotinic acid) and the other nucleotide is linked with adenine. The positively charged molecule results from the positively charged nitrogen of the pyridine ring. NAD+ and its reduced form NADH are used as a coenzyme for catabolic reactions. The reduced pyridine nucleotide NADH emits fluorescence peak at 450 nm upon UV excitation.

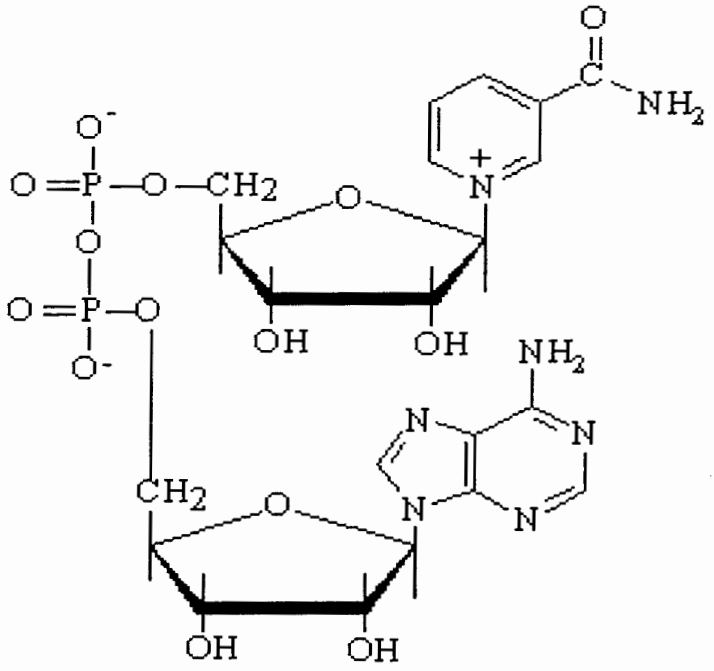

Figure 3 Structure of nicotinamide adenine dinucleotide (NAD^+).

The second pyrimidine nucleotide, FAD (Figure 4), is derived from the vitamin riboflavin and serves as a coenzyme for several enzymes including fatty acyl-CoA dehydrogenase, dihydrolipoyl dehydrogenase, succinate dehydrogenase, and alpha-glycerophosphate dehydrogenase. FAD containing an isoalloxazine ring (flavin nucleotides, indicated with a rectangle) is classified as the chromophore, while the

reduced form of FAD does not have fluorescence properties. However, oxidized FAD has an emission peak at 520–530 nm upon excitation at 440 nm (Juan Manuel Benavides 2003).

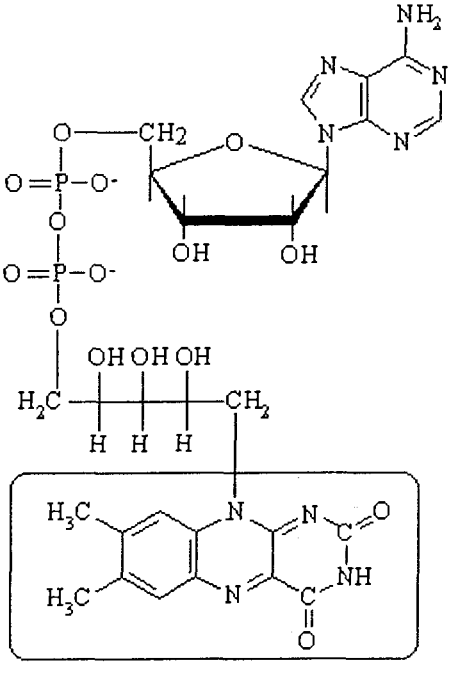

Figure 4 Chemical structure of flavin adenine dinucleotide (FAD).

Support for the involvement of NADH and FAD^+ in cellular autofluorescence comes from a recent study that used confocal microscopy to localize the source of autofluorescence in epithelial tissue. Ten pairs of colposcopically normal and abnormal cervical biopsies were examined by excitation with UV (351–364 nm) and visible light (488 nm). The fluorescence was localized to NADH and FAD^+ in mitochondria and the distribution of this cellular fluorescences was shown to be decreased strikingly with

degree of dysplasia (Pavlova *et al.* 2003). In addition, the contribution of these fluorophores towards the overall clinically visible fluorescence requires further evaluation. In contrast to increased FV from NADH and FAD^+, clinical observations suggested an overall lack of FV in oral precancerous lesions and cancer (Onizawa *et al.* 2002; Svistun *et al.* 2004).

I.8.4.2. Collagen fibres

Although NADH and FAD^+ contribute to the autofluorescence of normal oral and cervical mucosa as well as skin, the main source of autofluorescence was found to be due to the connective tissue collagen fibres and the cross-links of the collagen fibres (Pavlova *et al.* 2003). Collagen and their cross-links fluoresce upon excitation with either ultraviolet light or visible light of short-wavelength (e. g., around 488 nm). The changes in collagen fibres and their cross-links play a very important role in changes of autofluorescence of mucosa tissue.

Pavlova *et al.* (2003) looked at stromal autofluorescence in association with cervical dysplasia and showed that the fluorescence density of the stromal matrix immediately beneath the epithelium (the matrix density located 0.25~0.30 mm beneath the basement membrane) decreased as the tissue progressed from normal- to high-grade dysplasias. This finding suggested that the reduction of collagen fibres concentration was part of the tumourigenesis process, in which enzymes secreted by tumour cells or even precancerous cells degrade stromal collagen fibres. Another interesting study showed a 10-fold increase in the level of procollagen in the dermis immediately beneath the SCC of the skin. The level of procollagen is considered to reflect the activity of collagen

synthesis. This result supports the theory of dynamic changes (high turn-over rate) of collagen-dominant stroma immediately underneath the cancerous epidermis (Mitrani and Marks 1982).

I.8.4.3. Porphyrin

A final fluorophore that has been associated with autofluorescence in epithelial tissue is porphyrin. Porphyrin is a core component of haemoglobin, myoglobin, and cytochromes (Principles of Biochemistry, Lehninger *et al.* 1993). It is formed by the incorporation of iron atoms into protoporphyrin IX. Protoporphyrin IX is constructed from four molecules of the monopyrrole derivative porphobilinogen. Biosynthesis of porphyrin is regulated by the concentration of heme protein products, such as haemoglobin, which can serve as a feedback inhibitor of early steps in porphyrin synthesis. This biosynthesis pathway occurs in mammals, bacteria, and plants. Porphyrin compounds fluoresce in red upon excitation. Excitation–emission wavelengths for porphyrin compounds are 490 nm / 630 nm (Juan Manuel Benavides 2003), although 410 nm / 605–700 nm wavelengths may also be involved (Ingrams *et al.* 1997).

Red fluorescence can be seen normally on the dorsum tongue and dental plaques. During carcinogenesis, some tumours also emit red fluorescence, suggesting the presence of porphyrin. The origin of the porphyrin, however, remains speculative. It is possible that with tumourigenesis, there are accumulations of porphyrinogen intermediates that are excreted as porphyrins or porphyrin-related compounds (Harris and Werkhaven 1987; Onizawa *et al.* 2002). An alternative explanation is that some of the red fluorescence is a product of bacterial infection (Inaguma and Hashimoto 1999; Onizawa *et al.* 2003).

I.8.5. Light-blocking components (absorption and scattering) in oral tissue

I.8.5.1. Tissue absorption of light

All tissues in the body absorb light at various wavelengths, and this absorption process converts most of the light energy into heat. Exceptions are light that produce photochemical reactions or photons absorbed by fluorophores that produce fluorescence. At the same time body tissue, as any object above absolute zero temperature, generates light radiation know as black-body radiation. Thus, light can be used to heat tissue for therapy or be measured from tissue to determine temperature.

The absorption of energy as a result of an electronic transition causes an absorption band in a spectrum. The tissue components that absorb light are called chromophores, which are responsible for a UV or visible light absorption spectrum. If a molecule absorbs wavelengths ranging from 180 to 400 nm, an ultraviolet light spectrum will be obtained; if it absorbs wavelengths ranging from 400 to 780 nm, a visible spectrum is obtained. Whether a molecule can absorb light depends on the availability of non-bonded electrons within it. Molecules that are excited by light usually contain conjugated double bonds. Haemoglobin is a good example of an organic molecule that contains conjugated double bonds that result in multiple absorption peaks when exposed to the visible light spectrum (540-577nm). Figure 5 shows the chemical structure of haemoglobin after it combines with oxygen (oxy-haemoglobin). Figure 6 shows the absorbance spectrum for the molecule.

In the oral cavity, the main endogenous chromophore that absorbs light is haemoglobin. Oxy- and deoxy-haemoglobin is responsible for light absorption at

wavelengths below 600 nm and above1000 nm (Jacques 2001). Oxy-haemoglobin has absorption peaks that are below 420 nm, at 540 nm, and at 575 nm (Inaguma and Hashimoto 1999; Sciubba 1999). Due to the large amount of light absorption of haemoglobin, optical penetration beyond 1 mm from the sub- connective tissue layer is impractical in wavelengths below 600 nm. The focus of this study is the oral mucosal epithelium and the underlying lamina propria; they are both above the sub- connective tissue. The light energy can be propagated through these two layers ideally, and then absorbed by haemoglobin. However, in the event of increased vascularity in the superficial connective tissue layer, increased light absorption by haemoglobin will occur. Increased vascularity in the superficial connective tissue layer can occur both during increased angiogenesis (carcinogenesis) and during inflammation.

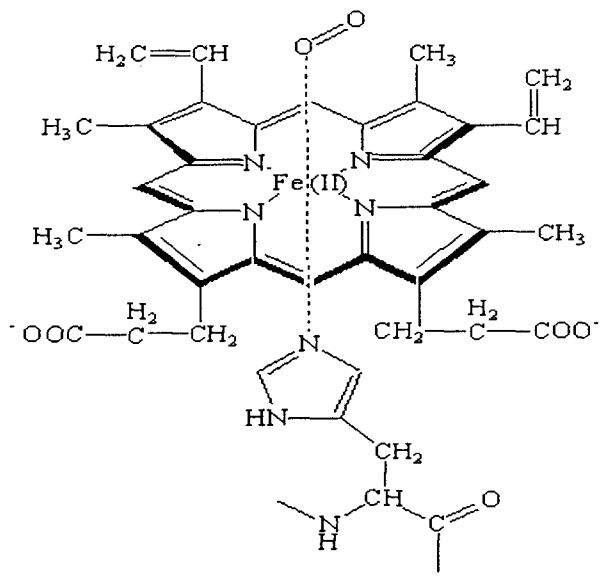

Figure 5 Chemical structure of oxy-haemoglobin.

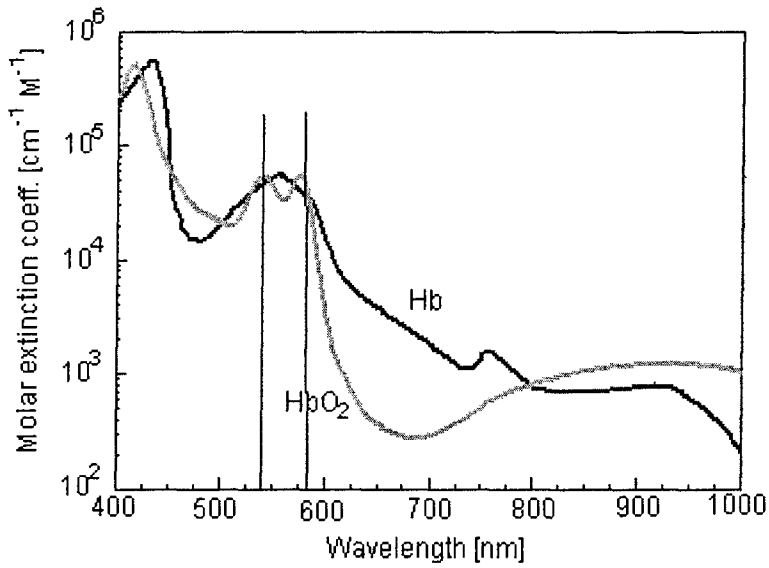

Figure 6 Absorbance spectrum of haemoglobin.

The figure demonstrates that minimal absorption for haemoglobin occurs between 650nm and 700 nm with peak absorptions at 540—780 nm (green-to-yellow region). (Adapted from ttp://www.medphys.ucl.ac.uk/research/borg/research/NIR_topics/nirs.htm)

I.8.5.2. Tissue scattering of light

Tissue scattering arises from the microscopic heterogeneity of refractive indices between extra-cellular, cellular, and sub-cellular components. The scattering properties as a result of changes in cell morphology, size, and shape have been critically evaluated in several kinds of tissues. The following tissue scattering factors will be discussed:

Mitochondria

Mitochondria occupy 22% of the hepatocyte volume, and are believed to be the predominate component for light scattering in the liver (Beauvoit *et al.* 1994). Studies show that carcinogenesis process in general accompanied by a remarkable increase in the

amount of mitochondria in the epithelial cells. Thus, increased mitochondria will cause more light scattering for both incidence and FV, which may show a decrease in intensity of light from tissue.

Nuclei

It is well known that nuclei can scatter light and that nuclei number, size and hyperchromatism will increase in the carcinogenesis process. Current literature supports that these factors should result in an increase in light scattering and decrease in FV in mammary and colorectal tumours, oral cancers, and OPLs. (Gurjar *et al.* 2001; Troy 1996). These results are supported by a further study of *in vivo* scattering measurements in OPLs and cancer tissue, that showed a correlation with the percentage of cells containing an enlarged nucleus (Sciubba 1999).

Cell size

A study also demonstrated the effect of cell size on light scattering in perfused liver samples (Chance *et al.* 1995). It suggested that solute concentrations may impact the scattering coefficient either by altering the osmotic pressure and thereby cell sizes, or by changing the relationship of refractive index between scatterers. But another group suggested that the changes of scattering properties were due to the relative volumetric ratio of cytoplasm to nucleus rather than actual cell size (Sloot *et al.* 1988).

Tissue surface

Increased roughness of the tissue surface will result in increases in light scattering in both excitation and emission pathways. The direction of light travel within the tissue medium (such as the epithelium and the connective tissue) depends significantly upon the

incidence angle of excitation light entering the tissue medium (air–tissue interface) (Richards-Kortum and Sevick-Muraca 1996). In other words, excitation light travels differently if it illuminates the tissue surface perpendicularly or obliquely. For example, both an ulcerated lesion and non-homogenous lesion of the oral mucosa share the common feature of losing the smooth mucosa surface. The increased roughness of an ulcer and non-homogenous lesion could attenuate the propagation of light. More importantly, the light scattering effect is clinically different in between macroscopically rough surfaces from and adjacent 'smooth' surfaces clinically. Similar scattering phenomena also arise at the interface between cellular and extra-cellular compartments due to the different refractive indices.

Stromal arrangement

A study also demonstrated the effect of some cellular and extra-cellular molecules towards light scattering and reflection in human tissues (Lynn *et al.* 1993). It suggested that tissue scattering increased as the number and size of collagen fibres in the dermis increased. The latter suggests that structures in the stroma may also play a role in tissue scattering. Whether the scattering effect from collagen fibres significantly affects the availability of their fluorescence emission signal at the tissue surface has yet to be measured.

I.8.6. Fluorescence visualization (FV) as an adjunct tool in identification of malignant and premalignant lesions

Since molecular and histological changes in premalignant and malignant lesions precede the clinical changes, autofluorescence examination offers a potentially powerful

tool in the identification of early high-risk lesions. Studies have shown that FV could identify precancerous and cancerous lesions in various organs including the oral cavity.

I.8.6.1. FV as a tool for the identification of precancerous and cancerous lesions in other organ

FV in cervical and esophageal lesions

Benavides published a study that focused on the development of a cost-effective *in vivo* imaging tool to detect cervical pre-cancer lesions (Juan Manuel Benavides 2003). The device was used to ascertain fluorescence emission patterns in a range of tissue types, including samples from 126 histologically normal sites, 31 high-grade squamous intraepithelial lesion site (SILS), 63 low-grade SILS, and 107 inflammatory and/or metaplasia sites. They used excitation wavelengths of 345 nm (UV) and 445 nm (visible light, similar to the wavelengths used this study). The data suggested a significant correlation between the reduced autofluorescence in green region and the pathology results. A classification algorithm was determined based on the fluorescence properties in three colour channels: red, blue, and green. This algorithm performed well for the discrimination of normal vs. high-grade SILS, and columnar normal-grade vs. low-grade SILS.

Another recently published article showed NAD(*P*)H and collagen have a potential to act as *in vivo* quantitative fluorescent biomarkers of epithelial precancerous changes (Georgakoudi *et al.* 2002). This article attempted to identify and adjust distortions that introduced in measured tissue fluorescence spectra by tissue scattering and absorption. Fluorescence and reflective spectra were simultaneously measured at sites from 35

patients with suspected cervical lesions and 7 patients with Barrett's esophagus. Biopsy samples of squamous metaplasias and high-grade squamous intraepithelial lesions (premalignancy) exhibited a 5-fold decrease in collagen fluorescence relative to normal squamous epithelium, and a 2-fold increase in the NADPH fluorescent contribution for the HSILS (precancerous lesion) compared with the benign squamous metaplasia site. The authors suggested that tissue deoxygenation, an increased number of cells, and increased levels of metabolic activity in epithelial cells could be responsible for the increase in NADPH content in precancerous tissue. The decrease in collagen might be attributed to the presence of collagenases such as matrix metalloproteinases, which are involved in the breakdown of collagen.

FV in lung and upper airway lesions

Lung imaging fluorescence endoscopy, or LIFE® (Xillix Technologies Corp.), is one of well-researched autofluorescence imaging systems for bronchoscopic evaluation of clinical abnormalities in the lung and upper airways. This system employs a helium-cadmium laser light (442 nm) for tissue excitation and two image-intensified CCD cameras to record the tissue fluorescence signals. The ratio of (enhanced) red to (reduced) green signals are computerized and compared with a predetermined value. A pseudocolour image that corresponded to the suspected cancer sites appears on a video monitor, to assist the clinician in determining which tissue requires further follow-up. This device has been used by numerous clinics worldwide as a resource to guide biopsy collection (Delank *et al.* 2000; Kulapaditharom and Boonkitticharoen 1998; Lam *et al.* 2000). The device has also been used, although less frequently to complement

microlaryngoscopy and assist clinicians in detecting and delineating laryngeal malignancies (Delank *et al.* 2000; Zargi *et al.* 2000).

FV in brain lesions

The excitation–emission matrix of a few human brain tissue samples was examined using ex vivo techniques in order to determine the feasibility of identifying gliomas with this approach. Three excitation–emission wavelength pairs were chosen to compare normal and tumour tissues: 360 nm / 470 nm (to detect NADH alterations), 440 nm / 520 nm (flavin), and 490 nm / 630 nm (porphyrin). Fluorescent emissions of NADH were lower in human brain tumours than in normal brain tissues. In contrast, flavin and porphyrin emissions varied in intensity according to tumour types (Chung *et al.* 1997).

FV in skin lesions

Several studies evaluated autofluorescence as a non-invasive aid to skin cancer detection (Andersson-Engels *et al.* 2000; Na *et al.* 2001; Panjehpour *et al.* 2002; Wang *et al.* 2003). Among them, the Panjehpour article is one of the more extensive studies. It measured the overall intensity of skin fluorescence emission using short-wavelength (410 nm) visible light in 279 skin sites from 49 patients. The results suggested that basal cell carcinoma and squamous cell carcinoma exhibited a fluorescence emission that was weaker than that of normal and premalignant sites (FV Loss, FVL). Retrospective data were used to establish a fluorescence intensity threshold and to assess the prospectively collected data. As a result, 90% of the cancer sites and 82% of the normal sites could be correctly discriminated using this threshold. The author attributed the reduction of fluorescence intensity in skin cancer sites to the absorption of fluorescent emission from

increased blood supply and from melanin in tumour sites and to the destruction of collagen and elastin cross-links at tumour sites.

Another article is also interesting in that the author attempted to use this approach to demarcate the clinically invisible margin of skin basal cell carcinoma using autofluorescence intensity (Na *et al.* 2001). Complete removal of this tumour is important, since recurrence was reported for 30-67% of cases (Rippey and Rippey 1997; Sigurdsson and Agnarsson 1998; Sussman *et al.* 1994). Twenty-one lesion sites were measured with fluorescence intensity from the lesion centre outwards. A loss of fluorescence intensity could be found up to 10 mm away from the clinical margin. Although these results were inconclusive (there is no indication of outcome for cases with different margins, they did support the possibility of using FV for such a purpose.)

I.8.6.2. FVL oral lesions from hamster oral cancer model

One of the most commonly used animal models for the study of oral cancer development is the 7,12-dimethylbenzanthracene (DMBA)-induced hamster buccal pouch carcinogenesis model (Parkin *et al.* 2005). The association of alterations with autofluorescence and the progression through premalignancy to cancer have been studied extensively in this model, using both ex vivo and *in vivo* analysis (Balasubramanian *et al.* 1995; Chen *et al.* 1998; Wang *et al.* 1999). In general, these studies all supported the potential value of using autofluorescence spectroscopy to detect pre-cancerous and cancerous lesions, with data showing that the approach is capable of discriminating between specimens of normal mucosa / hyperkeratosis, dysplasia, and carcinoma with a high degree of accuracy.

Of these studies, one by Wang *et al.* (2003) clearly supported a potential role for FV as a diagnostic tool, because a sensitivity of 92% and a specificity of 95 % were observed for differentiating benign (normal or hyperkeratosis) from premalignant (dysplasia) or malignant sites.

FV revealing increased porphyrin red fluorescence in oral lesions from hamster oral cancer model

A study was conducted to analyze the spectral properties of the red fluorescence observed in human and experimental animals and to spectrometrically establish the involvement of porphyrin production in that fluorescence (Onizawa *et al.* 2002). The study used an animal model that used 9,10-dimethyl-1, 2-benzanthracene application to the lateral border of the hamster tongue to induce tongue carcinomas. Fluorescent samples were obtained from 18 hamsters after excitation at 404 nm and compared with samples from 5 patients with SCCs (all emitting red/orange fluorescence after excitation), and with cell media collected from 3 human oral SCC. The contralateral, untreated border of the tongue of six hamsters served as controls. Ten mild dysplasia, five severe dysplasia and three SCC cases were analyzed from the hamster samples. Red/orange fluorescence was observed in all 3 experimentally induced SCC, in 1 of 10 mild dysplasia, and 1 of 5 severe dysplasia. All controls were negative for fluorescence. The spectral profile of the experimentally induced cancers changed with malignancy, with increases in intensity at 634 nm and 672 nm and decreases at 582 nm. Porphyrin peak intensity at 634 nm and 672 nm increased as the severity of pathology progressed from control, to mild dysplasia, to severe dysplasia, and to SCC; however, the difference was significant only for intensity changes from control, hyperplasia, or early cancer to

invasive cancer. Of specific importance was the observation that the ratios of the intensity values of 582 nm over 634 nm might be of diagnostic utility in differentiating between non-malignant and malignant tissues. These values were consistently altered not only in experimental carcinomas but also in the clinical cancer samples and cultured cancer cells. Furthermore, the study showed that the oral cancer cell itself can produce porphyrin and that its accumulation in tumour tissue produces red fluorescence under UV excitation.

I.8.6.3. FV as a tool for the identification of human oral SCCs

The delineation of oral carcinomas is usually not an easy task because: 1) Tumours usually start out as surface lesions which spread over the surface of mucosa and sometimes merge together with surrounding inflammation or leukoplakia; 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, colour 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; and 5) Due to the unique characteristic of multicentric origin of oral cancer, it is not unexpected for multiple or satellite tumours to exist adjacent to the main tumour mass and these are often missed during clinical examination (Strong *et al.* 1968).

The following is a summary of several the key publications that describe the use of FV to detect or delineate oral SCCs (Table 2).

Table 2 Use of FV to identify oral SCC and OPLs

Reference	No. of patients	Excitation/emission wavelengths (nm)	Study target	Sensitivity (%) / Specificity (%)	Autofluorescence or FV results
Kolli *et al.* 1995	19	290, 330/(380) 330/(390, 450)	Oral SCC and Epithelial thickness	NA*/NA*	FVL associated with SCC. Increased epithelial thickness is associated with FVL.
Onizawa *et al.* 1996	34	360	Porphyrin and SCC	94/96	Increased orange fluorescence from protoprophyrin correlated with tumour vs. benign and dysplasia.
Fryen et. al. 1997	89	Unclear	The upper aerodigestive tract SCC	71/NA*	Increased fluorescence from elastin was showed in not clearly visible tumours.
Kulapaditharom *et al.* 1998	11	442/(green, red)	Oral SCC	100/87. 5	FVL associated with lesions from normal, dysplastic, and malignant oral mucosa.
Schantz *et al.* 1998	35	300–340/(450)	NADH activities and cell differentiation	NA*	NADH fluorescence positively correlates with grade of tumour. Fluorescent signal may predict the potential for recurrence. Cell differentiation was associated with FVL.
Gillenwater *et al.* 1998	15	337, 365, 410/ (Red/blue)	Comparing flavin, NADH, collagen in precancer, cancer and control	100/83	FVL as a result of increased red fluorescence in lesions vs. control Inter-individual and intra-individual variation of FV status.

Reference	No. of patients	Excitation/emission wavelengths (nm)	Study target	Sensitivity (%) / Specificity (%)	Autofluorescence or FV results
Onizawa *et al.* 1999	130	360	Porphyrin and SCC	94/96	Increased orange fluorescence from protoprophyrin correlated with tumour vs. benign and dysplasia
Betz *et al.* 1999	30	375–440/(green)	FAD, elastin, porphyrins in SCC	NA*	FVL was showed in SCC as compared to adjacent tissue
Inaguma *et al.* 1999	78	410/(> 600)	Porphyrin and SCC	100/96	FVL as a result of increased red porphyrin-like fluorescence in oral SCC but not in normal mucosa
Onizawa *et al.* 2003	55	360/(red)	Porphyrin and SCC	90. 9/ NA*	FVL as a result of increased red porphyrin-like fluorescence correlated with tumour and tumour staging
Muller *et al.* 2003	15	337, 358/(~ 450)	Fluorophores (NADH, collagen) Scattering by cell differentiation Scattering by kerantinization	83/96	FVL increased with progression resulting from: Increased NADH and decreased collagen with progression Increased scattering from nuclei size growing bigger with progression Increased scattering from hyper-keratinisation

NA*: Not available

I.8.6.4. FV as a tool for the identification of human OPLs

There has been far less research conducted on the efficiency of FV in detecting human premalignant lesions in contrast to studies on oral SCC.

Ingrams performed a small study with 11 patients attending an otolarynology–head and neck surgery clinic to determine whether fluorescence spectroscopy could differentiate normal and dysplastic tissues *in vitro* and to obtain information on the optimal excitation wavelength for identifying dysplasias in future *in vivo* studies (Ingrams *et al.* 1997). Patients were identified as having clinically suspicious oral mucosal lesions. Biopsies were taken from abnormal and normal regions and examined spectroscopically. On histological examination, 10 specimens were classified as abnormal (this included dysplastic or malignant diagnoses, unfortunately, with no indication of relative numbers of each) and 12 samples were classified as normal (healthy or with benign changes). Significant differences were seen between the 2 groups with the most striking observed at the excitation wavelength of 410 nm, where 20 or 22 samples were correctly identified as abnormal by fluorescence red spectrum. The increased red fluorescence in dysplastic and cancer tissues occurred with emission peaks above 600 nm with the most prominent peaks at 635 nm and 690 nm, which were not seen in the corresponding normal tissue. The ratio of prominent peak intensity (640 nm / 615 nm) and the area under the curve from 600 nm to 650 nm for normal and dysplastic (or cancer) lesions was used to establish a cutoff line. Using this algorithm, the sensitivity and specificity to differentiate histologically normal from dysplastic and cancerous tissues were 90% and 91%.

Muller used tri-modal spectroscopy to assess reflectance, light scattering, and fluorescence in 15 patients with known upper aerodigestive tract cancers, including 12 patients with cancer in the oral cavity (Muller *et al.* 2003). The objective was to determine how well such spectral features correlated with early biochemical and histological change. Spectral data were collected from 91 tissue sites in these patients with 53 biopsies of varying histology (16 biopsies were normal, 11 mild/moderate dysplasia, 8 severe dysplasia, 12 SCC, 2 inflammation, and 1 keratosis). These spectral data were compared to the data obtained from 38 tissue sites, none of which were biopsied, in 8 healthy volunteers. Diagnostically significant information was found to lie in the emission wavelength range of 350–600 nm, where intrinsic fluorescence of oral cavity tissue comprised collagen and NADH. Based on the spectral data, NADH appeared to increase with disease progression and collagen appeared to decrease. Reflectance spectra suggested that the scattering phenomena became prominent with the extension of dysplasia and the enlargement of epithelial cell nuclei. Scattering increased with keratinization, higher in normal keratinized mucosa than non-keratinized mucosa. The researchers concluded that this combination of spectroscopy could distinguish malignant and precancerous tissues from normal tissue with a sensitivity and specificity of 96%. The diagnostic sensitivity for fluorescence is calculated to be 83% for cancer and 53% for OPLs using the raw data supplied in the article.

Finally, Gillenwater used fluorescent spectroscopy to collect data from the oral cavities of 8 healthy controls and 15 patients with premalignant or malignant oral lesions (Gillenwater *et al.* 1998). Patients attended a tertiary care head and neck cancer clinic. Histological diagnosis included 15 normal, 5 reactive atypia, 12 dysplasia, 4 *CIS*, and 9

cancers (45 in total). There was no indication whether patients with dysplasia had a prior history of cancer or of preceding treatment for any of the cases. Data from 3 patients were later excluded due to instrument error, leaving 12 patients with 33 sites for analysis. Spectral data were collected with 3 excitation wavelengths (337 nm, 365 nm, and 410 nm) and an emission range of 350~700 nm and were used to develop diagnostic algorithms. Peak fluorescence intensity for the spectra showed both inter-individual and intra-individual variations. Of interest, there were variations in the same individual for contralateral sites. The person-to-person variation at each site was greater than the contralateral variation within a particular site in the same person and the variation among different anatomical sites within the same person. For the 3 excitation wavelengths assessed, the fluorescent emission intensity ratio of red to blue showed less variation between healthy sites and individuals compared with the absolute peak intensity. In addition, the excitation wavelength of 410 nm showed the least variation in intensity ratio. Compared with the fluorescence spectrum on the contralateral sites, the intensity of the blue peak was higher in a normal site, and that of the red peak was higher in an abnormal site. Red-to-blue (635 nm / 490 nm) peak ratios were greater in abnormal tissue than at a contralateral normal site within the same patient. By establishing a cutoff line (+ 1 SD) in the peak intensity and the red-to-blue ratio of healthy sites, the sensitivity and specificity of differentiating healthy from dysplastic or cancer sites was determined to be 94.1% and 100% respectively.

I.8.6.5. Autofluorescence in other human oral lesions

In addition to exploring the autofluorescence of cancers, Inaguma and Hashimoto (1999) also evaluated autofluorescence in 43 other patients with oral lesions diagnosed as benign based on clinical appearance and history (Inaguma and Hashimoto 1999). The assessment again was for porphyrin-like fluorescence. The set included 15 leukoplakia (no histological diagnosis given), 3 lichen planus, 17 inflammatory disease, 2 pigmentation cases, and 6 benign tumours. Only 1 case of acute necrotizing ulcerative gingivitis and 2 of the leukoplakia showed porphyrin-like fluorescent emission.

Chen observed the fluorescence spectrum of oral submucous fibrosis upon excitation at 330 nm (Chen *et al.* 2003). Oral submucous fibrosis is a chronic scarring disease characterized by epithelial atrophy and progressive deposition of collagen in the lamina propria and submucosa of the oral mucosa. This mucosal condition usually results from the chewing of the areca quid. An increase in emission at 380 nm (which corresponds to collagen) was observed for the fibrotic tissue compared to normal tissue. Emission at 460 nm (corresponds to NADH) was decreased. These profiles are in contrast to those reported for cancer, where emission is decreased at 380 nm and increased at 460 nm.

I.8.7. Direct fluorescent visualization (FV)

Instrumentation for fluorescence imaging requires a light source, excitation and emission filters, and a means of detection. The fluorescence can be detected and visualized directly by a human observer, or recorded by a camera and visualized

indirectly. The promise of optical technology has been shown by increasing numbers of studies, including studies demonstrating alteration of autofluorescence in oral malignancies and premalignancies (see above). However, these studies have mostly employed complex devices to measure spectroscopy indirectly – using either photographic film or a sensitive or intensified charge-coupled device camera (Betz *et al.* 1999; Onizawa *et al.* 2003; Richards-Kortum and Sevick-Muraca 1996).

Unlike the previous studies, our research group has developed a simple hand-held device called FV that can be used to directly visualize changes in the autofluorescence (Lane *et al.* 2006).

I.8.7.1. Advantages of direct FV as a tool in identifying OPLs and SCC

Direct FV is an exciting novel technology in oral oncology and has some distinct advantages:

It is non-invasive in nature: Such a visual tool does not involve chemicals and has no potential side effects. It is not a dye, so it leaves no messy stains. It can be used to scan relatively large tissue areas, such as the whole oral cavity, without relying on the clinical identification of a lesion.

Simple: FV, the simple hand-held device, does not require complicated instruments or equipments and can be easily employed in a private office of clinicians.

Because of the non-invasive, simple-to-use nature of the device, it has the potential to be used in secondary prevention of oral cancer. Primary prevention involves the identification and elimination of exposure to cancer-causing agents and habits. This

would involve altering alcohol and tobacco use for oral cancers. Secondary prevention is accomplished through multi-stage screening programs that are used to identify patients at elevated risk to allow time to intervene early in the progression of the disease. Over the last couple of decades, there has been an increasing emphasis on the latter approach, largely due to the success of the PAP smear screening program in decreasing cervical cancer incidence and morality. Medical and technological advances have created many new screening approaches that have yet to be evaluated. In order for such procedures to be effective and accepted by the public, they must be cost-effective, non-invasive and easy to perform.

So far there are no available screening technologies for oral cancer in high-risk populations. Exfoliative cell technology like that of the PAP smear does not work in the oral cavity. OPLs tend to be more keratinized than lesions at uterine cervix, hence smears from an oral lesion tend to show squames without nucleated cells for diagnosis, leaving a false negative result. More importantly, as a screening tool, the whole oral cavity should be screened. However, taking smears of the whole oral cavity is not practical.

Thus FV can potentially be a screening tool for the whole oral cavity and a tool for secondary prevention.

Other visual tools, such as toluidine blue, have shown promise in the identification of oral lesions. The availability of different visual tools are important not only because cancer development involves different pathway and requires different visual tools, but also because of the need for visual tools of different specificity and sensitivity. One of

the invariable characteristics of visual aids or markers for high-risk lesions is that the ones high in specificity are low in sensitivity, and vice versa. The availability of visual aids that have different sensitivities and specificities would be advantageous in the clinical diagnosis since the visual aids can complement each other.

I.9. Direct fluorescent visualization examination

The following describes the procedure for use of FV in direct FV examination. This part has been done by Dr. Samson Ng, a dentist in our research group, for his MSc thesis, and forms the basis for my study (Ng, 2005).

For autofluorescence examination, an external light source is used to illuminate the target tissue (the excitation light). Target tissue fluoresces under the excitation light and the light produced by the tissue itself is called autofluorescence (a colour different from the excitation light). In order for the examiner to see the autofluorescent light only, a filter is needed to block out the colour of the excitation light. This is accomplished by the examiner wearing a pair of glasses or goggles with the filter.

In Dr. Samson Ng's study, the excitation light was blue and violet in colour and was produced by a halogen arc lamp in a device called SCD-1 from Biomax to examine the oral mucosa of all test subjects. An integrated parabolic reflector collected the light from the light bulb and produced a homogenous output beam through a diffuser. The wavelength of the excitation light was in the range of 400–460 nm using 460 nm bandpass filters centred at 425 nm (Chroma Technology Corp., USA). The measured

illumination intensity at the usual working distance (3–6 inches) was approximately 1–4 mW/cm^2 at 440 nm excitation.

The oral mucosa autofluorescence induced by the extraoral excitation light was green and red in colour. The examiner viewed the autofluorescence by wearing a pair of goggles or glasses with a long pass filter and a notch filter to allow only the passage of green (475–560nm) and red (620–740nm) fluorescent emission (Schott Glass Technologies, USA).

The FV examination was conducted in the absence of ambient light, at least five seconds after ambient light was turned off and excitation light was turned on.

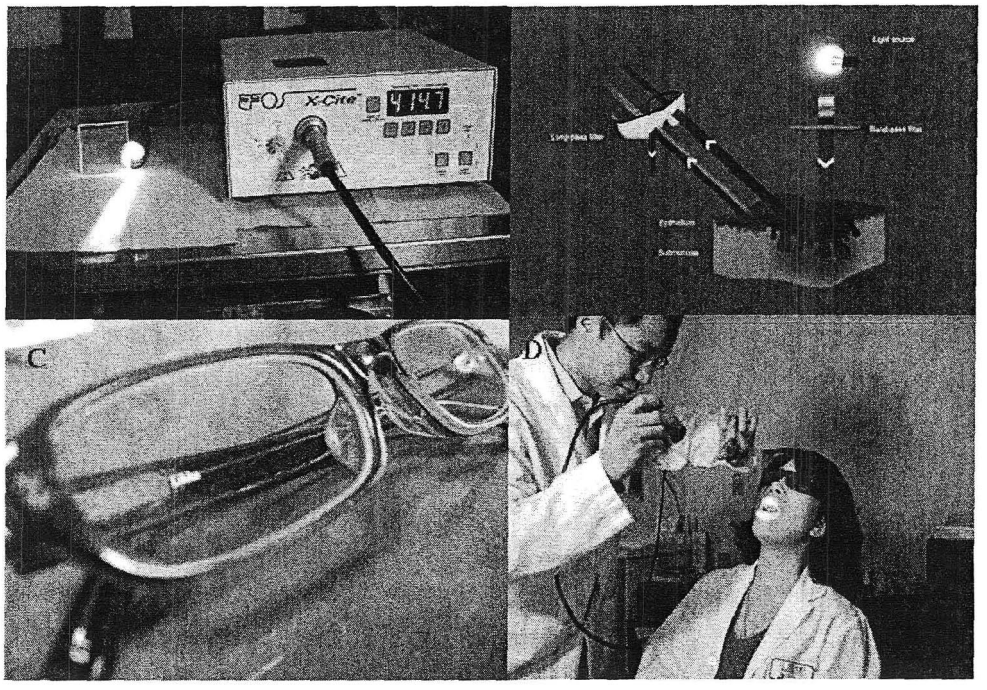

Figure 7 Direct fluorescence visualization (FV) technique

(A) Light source of excitation; *(B)* Illustration of direct fluorescence visualization (FV) technique;*(C)* Eye goggles with filters; *(D)* A clinician is examining patient's oral cavity by FV.

II. STATEMENT OF PROBLEM

Oral SCC is believed to be a multi-step process. The neoplastic process is believed to progress through hyperplasia to increasing degrees of dysplasia (mild, moderate and severe) to *CIS* and finally to invasive SCC. It is well established that accumulations of genetic alterations are the basis for cancer progression (Braakhuis *et al.* 2004). Once invasive cancer is formed, the prognosis is very poor (5 year survival rate $\leq 50\%$). Early detection and proper management of OPLs is one key to improving this dismal prognosis.

Only a very small percentage of OPLs progresses into cancer. Thus, distinguishing high-cancer-risk OPLs from low-risk OPLs is critical for the management of these lesions. Histology, the current gold standard for cancer risk assessment of OPLs, and more-recent molecular markers both rely on accurate biopsy samples of the OPLs. If clinicians fail to take a sample from a high-risk lesion, or take a sample but miss the highest cancer risk area, both histology and molecular cancer risk assessment can be useless.

Current decisions of when and where to biopsy are based upon identification of clinical risk factors of the OPLs. The most common method of screening for oral cancer is visual inspection for the clinical risk characteristics (Kujan *et al.* 2005). However, not only the clinical risk features could be easily confused with reactive or inflammatory changes of no malignant potential, but there also is ample evidence from both histological and molecular studies suggests that histological and genetic changes are not necessarily

associated with clinically observable morphological changes within the mucosa. Thus development of visual aid that could facilitate the clinical identification of high-risk OPLs is highly needed.

FV as an emerging advanced technology for cancer screening has attracted intense interest as a visual aid because it is non-invasive, simple to apply and does not involve chemicals. The literature has shown FV to be effective in the diagnosis of cancer in a number of organs including the lung, cervix, skin and oral cavity. Limited studies of FV have also been done on premalignant lesions. However, these studies have mostly employed complex devices to measure spectroscopy indirectly – using either photographic film or a sensitive or intensified charge-coupled device camera. There are also no studies, to the best of my knowledge, that have investigated the relationship between FV and molecular changes, or between FV and outcome of OPLs. This thesis is the first study to investigate the value of FV, a simple hand-held visual aid that could directly visualize tissue autofluorescence, in detecting high-risk OPLs through not only pathological studies but also molecular studies and outcome.

III. OBJECTIVES

1. To characterize the pattern of genetic changes of a group of oral lesions that have been examined with FV (FVL or FVR) by means of LOH analysis using 19 microsatellite markers for 7 chromosomal regions known to be frequently lost in oral tumours, and high-risk OPLs: 3p14.2 *(D3S1234, D3S1228, and D3S1300),* 4q26 *(FABP2),* 4q31.1 *(D4S243),* 8p21.3 *(D8S261),* 8p23.3 *(D8S262),* 8p23.3 *(D8S264),* 9p21*(IFNA, D9S171, D9S1748, and D9S1751),* 11q13.3 *(INT2),* 11q22.3 *(D11S1778),* 13q12.3 – 13 *(D13S170),* 13q14.3 *(D13S133),* 17p11.2 *(CHRNB1),* 17p13.1 *(tp53 and D17S786).*

2. To compare the histological gradings, LOH patterns and outcomes of FVL oral lesions with those of FVR cases.

3. To determine whether fluorescence visualization (FV) can identify OPLs at risk of cancer progression as judged by histology, molecular markers (LOH pattern) and outcome.

IV. HYPOTHESES

FVL oral lesions will show increased cancer risk as judged by histology (higher degree of dysplasia), molecular markers (high-risk LOH patterns) and by outcome (increased cancer progression) as compared to FVR cases.

If data support this hypothesis, it would suggest that FVL OPLs have a higher risk of progression than the FVR OPLs and would also suggest that FV status would not only be useful in identifying SCC and *CIS*, but also useful in identifying high-risk dysplasia or OPLs.

V. MATERIALS AND METHODS

V.1. Sample collection

This study was focused on OPLs. Patients with a history of histologically confirmed oral dysplasia or oral SCC were recruited from the "Oral Health Study", an ongoing National Institute of Dental and Craniofacial Research (NIDCR) - funded prospective study in the Oral Dysplasia Clinic at British Columbia Cancer Agency. These patients were primarily referred by community dentists or the Head and Neck Oncology Group at the British Columbia Cancer Agency (BCCA) for assessment and care. All clinical examinations were conducted in the division of Oral Oncology/Dentistry at the Agency during the study period from April 2002 to September 2005. Subsequent histopathological reviews were conducted in the Provincial Oral Biopsy Service at the Vancouver Hospital and Health Sciences Centre. The subjects were informed of the study procedures, and their informed consent was received.

The criteria for selecting cases included:

1.) Patients had consented to assessment as part of our longitudinal study at BCCA

2.) Patients had FV result determined with FV device for the study biopsy or index lesion

3.) The index lesion had histological diagnoses of hyperplasia, dysplasia, or *CIS* but not invasive SCC. These diagnosis were confirmed by two pathologists (Dr. L. Zhang and Dr. C. Poh, the Provincial Oral Biopsy Service, the University of British Columbia) using World Health Organization (WHO) diagnostic criteria.

The biopsy sample had sufficient DNA for the multiple LOH analysis.

The index lesion was followed for at least 6 months after the biopsy.

V.2. Sample sets

Sixty-one patients had 91 oral biopsies that had been examined with FV immediately prior to the biopsy procedure, and were classified as either FV retained (FVR, n = 35) or FV loss (FVL, n = 56).

V.3. Patient information

The following patient information was obtained from patient databases and given to me in a coded fashion (i.e. without any patient identifiers): demographic and habit data (age, gender, ethnicity, and smoking habits of the patients), pathological diagnosis, fluorescence status and outcome of the lesion (i. e. whether the lesion progressed into invasive cancer).

V.4. Slide Preparation

All formalin-fixed biopsies were embedded in paraffin blocks. A 5 μm thick section was cut from each block and stained with hematoxylin and eosin (H&E) as a

dissection reference slide while more sections were cut at a 10 to12 μm thickness for dissection. The number of sections cut for dissection depended on the size of each biopsy. H&E stain also applied to these slides. The H&E procedure is described below:

Slides were baked at 37°C overnight in an oven, then at 60° to 65°C for 1 hour, and left at room temperature to cool. Samples were deparaffinized by two changes of xylene for 10 minutes each, and then were hydrated in gradient ethanol (100%, 95%, and 70%) for 1 minute each, and rinsed in tap water. The slides were then placed in Gill's Hematoxylin for 5 minutes, then rinsed in tap water until the water was clear. The samples were blued with 1.5% (w/v) sodium bicarbonate then rinsed in water. Slides were lightly counterstained with eosin for 20 seconds then rinsed in tap water until the water was clear. The thick sections (10 to12 μm thick) were left un-covered in slide boxes and submitted for microdissection, whereas the 5 μm H.E. slides went to the next step. The later sections were dehydrated in gradient ethanol (70%, 95%, and 100%) for 1 minute each, placed in two changes of xylene for 10 minutes each and cleared, and coverslipped.

V.5. Microdissection

All diagnoses were confirmed by two pathologists using criteria established by the WHO. All epithelium of lesions were meticulously microdissected to isolate cells of hyperplasia, dysplasia, *CIS*, or tumour into different tubes. The underlying stroma was also collected as a source of matched control DNA. All samples were coded so that loss of heterozygosity (LOH) analysis was done without knowledge of diagnosis.

V.6. Sample Digestion and DNA Extraction

The microdissected tissue was digested in 300 μl of 50 mM Tris-HCL (pH 8.0) containing 1% SDS and 0.5 mg/ml proteinase K. All samples were incubated at 48°C in a water bath for 72 hours, and 10-20 μl of fresh 20 mg/ml proteinase K were added to the samples twice per day while incubated.

The Genomic DNA was extracted by phenol-chloroform mixtures (PC-9), and precipitated with 100% ethanol in the presence of ammonium acetate and glycogen, and washed with 70% ethanol. The DNA pellets were re-suspended in appropriate amount of LoTE (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA), dependant upon amount of tissue present in initial microdissection.

V.7. DNA Quantification

Sample DNA was quantified fluorometrically with a Picogreen kit (Molecular Probes) using an SLM 4899C spectrofluorometer (SLM Instruments Inc., Urbana, IL). The amount of sample DNA was determined using standard curves created by standard DNA. The final concentration of DNA working stock was diluted to 40 ng/μl with LoTE Buffer.

V.8. Primer-Extension Preamplification (PEP)

The DNA samples of less than 200 ng were amplified by primer-extension pre-amplification (PEP), which amplifies multiple sites of genome using random primers. PEP is in a 60 μl reaction system, containing 20 ng of the DNA sample, 900 mM of Tris-

HCL of pH 8.3, 2 mM dNTP , 400μM of random 15-mers (Operon Technologies), and 1μl of Taq DNA polymerase. PCR was performed in an automated thermal cycler (Omigene: Steinfurt, Germany). The amplification protocol consisted of one cycle of pre-heat at 95°C for 2 minutes, followed by 50 cycles of: (1) denaturation at 92°C for 1 minute, (2) annealing at 37°C for 2 min, and (3) polymerization at 55°C for 4 min.

V.9. End-Labeling

The 50 μl mixture for endlabeling contained 38μl of sterile double distilled water, 5 μl of a 10x buffer for T4 polynucleotide kinase (New England BioLabs, Ontario), 1 μl of 10X 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 (1 μCi/μl, Amersham: NJ, USA). The labeling process was done by incubating at 37°C for 1 hour.

V.10. PCR Amplification for microsatellite analysis

A serial of microsatellite markers (Research Genetics, Huntsville, AL) were applied to LOH analysis, which mapped to the following regions: 3p14.2 *(D3S1234, D3S1228, and D3S1300)*, 4q26 *(FABP2)*, 4q31.1 *(D4S243)*, 8p21.3 *(D8S261)*, 8p23.3 *(D8S262)*, 8p23.3 *(D8S264)*, 9p21*(IFNA, D9S171, D9S1748, and D9S1751)*, 11q13.3 *(INT2)*, 11q22.3 *(D11S1778)*, 13q12.3 – 13 *(D13S170)*, 13q14.3 *(D13S133)*, 17p11.2 *(CHRNB1)*, 17p13.1 *(tp53 and D17S786)*.

The PCR amplification was carried out in a 10μl reaction mixture containing 4 ng of genomic DNA template, 1 ng of labeled primer, 10 ng of each unlabeled primer, 1.5

mM each of dATP, dGTP, dCTP, and dTTP, 0.5 units of Taq DNA polymerase (Life Techs: Ontario) and PCR buffers (16. 6 mM ammonium sulfate, 67 mM Tris pH 8.8, 6.7 mM magnesium chloride, 10mM mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl sulfoxide). The amplification reaction was run in the thermal cycler for one cycle of pre-heat at 95 °C for 2 min; 40 cycles of the following: denaturation at 95 °C for 30 seconds, annealing at 50-60 °C (depending on the primer used) for 60 seconds, and extension at 70 °C for 60 seconds; followed by final extension at 70 °C for 5 min.

The PCR products were then diluted with 8 μl loading buffer, separated by 7% urea-formamide-polyacrylamide gels electrophoresis, and visualized by autoradiography on films.

V.11. Scoring of LOH

Films were coded and scored without knowledge of sample characteristics for relative intensity of autoradiographic bands. The migration of PCR products on gels is dependent upon the size of DNA fragments. For informative cases, which yield two bands representing each allele, allelic imbalance (loss or marked reduction of one of the allelic bands by 50% intensity comparing to the normal control), was recorded as LOH. When there was no significant alteration in the relative intensity of the 2 bands, the sample was scored as retention. When only a single band was present on the radiogram, the sample was scored as non-informative. When DNA was sufficient, samples showing LOH were subjected to repeat confirmatory analysis with an independent PCR reaction.

V.12. Statistical analysis

Differences and associations between groups (e.g. FVL vs. FVR) were examined using either Fisher's exact test or Chi-square test for categorical variables, and t-test for continuous variables. All tests were two-sided. $P < 0.05$ was considered to be statistically significant.

VI. RESULTS

VI.1. Demographics and habits of patients

Data for individual patients and samples have been placed in the Appendix for reference. Table 3 summarizes the demographic data for all patients in this study. Patient age ranged from 39 to 89 years old, with a mean of 59, 52% were male, 80% were of Caucasian ethnicity and 66% were current or prior smokers.

FVL and FVR cases were compared for the aforementioned clinicopathological features. No significant association was observed between FV presentation and age, gender, ethnicity, follow-up time or smoking history (Table 3).

Table 3 Demographic features of patients

	All cases	**FVL cases**	**FVR cases**	***P value***
Number of lesions	91	56	35	
Mean age (year) ± SD	59 ± 13	60 ± 12	58 ± 13	*0.30*
Male sex - # (%)	47/91 (52%)	25/56 (45%)	22/35 (63%)	*0.13*
Caucasian Ethnicity - # (%)	73/91 (80%)	45/56 (80%)	28/35 (80%)	*1*
Tobacco use ever - # (%)	57/86 (66%)	33/54 (61%)	24/33 (73%)	*0.35*
Follow-up time (month) ± SD	27 ± 12	28 ± 12	26 ± 13	*0.33*

VI.2. FV and histology

All the oral lesions in this study are premalignant or preinvasive. According to the histological progression model, they are put into three categories: 1) Oral lesions without dysplasia (reactive hyperplasia or inflammation): 28 cases; 2) Oral lesions with low-grade (mild or moderate) dysplasia: 39 cases; 3) Oral lesions with high-grade dysplasia (severe dysplasia/*CIS*): 24 cases.

Table 4 examined the association between FV and the presence of dysplasia in the lesions. The results showed that the presence of dysplasia was accompanied by a significant increase in FV loss (43% of non-dysplastic lesions demonstrated FVL vs. 70% of the dysplastic lesions; $P = 0.02$). Table 5 examined the association between FV and the degree of dysplasia in the lesions. Again, there was an increasing proportion of the lesions demonstrating FV loss with increasing severity of the histology ($P < 0.0001$).

Table 4 Fluorescence visualization alterations and presence of dysplasia

	All	**Non-dysplastic lesions**	**Dysplastic lesions**	***P* value**
# of cases	91	28	63	
FVR	35	16/28 (57%)	19/63 (30%)	***0.02***
FVL	56	12/28 (43%)	44/63 (70%)	

Table 5 Fluorescence visualization alterations and the severity of dysplasia

	All	Low-grade dysplasia	High-grade dysplasia	*P* value
# of cases	63	39	24	
FVR	19	19/39 (49%)	0/24 (0%)	***< 0.0001***
FVL	44	20/39 (51%)	24/24 (100%)	

VI.3. LOH and histology

Table 6 examined the relationship between LOH and histology. Most categories of the comparisons showed an increasing frequency of LOH with increasing severity of histology from nondysplastic lesions to low-grade dysplasia to high-grade dysplasia. This trend of increase was significant for 6 of the 7 individual chromosome arms tested: at 3p (8% in non-dysplastic lesions, 27% in low-grade dysplasia, and 58% in high-grade dysplasia, P = 0.0004), at 4q (4% in non-dysplastic lesions, 32% in low-grade dysplasia, and 63% in high-grade dysplasia, P < 0.0001), at 9p (25% in non-dysplastic lesions, 59% in low-grade dysplasia, and 91% in high-grade dysplasia, P < 0.0001), at 11q (14% in non-dysplastic lesions, 13% in low-grade dysplasia, and 57% in high-grade dysplasia, P = 0.0002), at 13q (4% in non-dysplastic lesions, 11% in low-grade dysplasia, and 25% in high-grade dysplasia, P = 0.005), and at 17p (12% in non-dysplastic lesions, 44% in low-grade dysplasia, and 65% in high-grade dysplasia, P = 0.0005). The increase in the

severity of histology is also accompanied by an increase in the high-risk pattern LOH: for multiple loss (18% in non-dysplastic lesions, 56% in low-grade dysplasia, and 92% in high-grade dysplasia, P< 0.0001), and for LOH at 3p and/or 9p plus any loss on other arms (19% in non-dysplastic lesions, 46% in low-grade dysplasia, and 88% in high-grade dysplasia, P < 0.0001).

Table 6 LOH and histology

	All	Non-dysplastic lesions	Low-grade dysplasia	High-grade dysplasia	*P* value
# of lesions	91	28	39	24	
LOH on chromosome arm					
3p	26	2/26 (8%)	10/37(27%)	14/24 (58%)	***0.0004***
4q	27	1/28 (4%)	11/34 (32%)	15/24 (63%)	***< 0.0001***
8p	17	3/27 (11%)	10/39 (26%)	4/24 (17%)	*0.32*
9p	51	7/28 (25%)	23/39 (59%)	21/23 (91%)	***< 0.0001***
11q	22	4/28 (14%)	5/39 (13%)	13/23 (57%)	***0.0002***
13q	13	1/28 (4%)	4/37 (11%)	8/23 (25%)	***0.005***
17p	35	3/28 (12%)	17/39 (44%)	15/24 (65%)	***0.0005***
High-risk LOH patterns*					
>1 arm lost	49	5 (18%)	22(56%)	22 (92%)	***< 0.0001***
p &/or 9p plus others	43	5/26 (19%)	17/37 (46%)	21/24 (88%)	***< 0.0001***

*(Rosin *et al.* 2000)

In Table 7 a determination was made of the point in the progression of the lesions at where change in LOH frequency became significantly different. When individual chromosome loci were examined, 3 of the 7 chromosome arms (4q, 9p and 17p) showed a significant increase in LOH frequencies in low-grade dysplasia as compared to the nondysplastic lesions; and 5 of the 7 chromosome arms (3p, 4q, 9p, 11q and 13q) showed a significant increase in LOH frequencies in high-grade dysplasia as compared to the low-grade dysplasia. When high-risk LOH patterns were examined, significant differences were noted for both multiple losses and for LOH at 3p &/or 9p plus others between non-dysplastic lesions and low-grade dysplasia and between low-grade dysplasia and high-grade dysplasia.

Table 7 Association of LOH with histological diagnosis

	All	Non-dysplastic lesions	Low-grade dysplasia	*P value*[1]	High-grade dysplasia	*P value*[2]
# of lesions	91	28	39		24	
LOH on chromosome arm						
3p	26	2/26 (8%)	10/37 (27%)	*0.10*	14/24 (58%)	***0.02***
4q	27	1/28 (4%)	11/34 (32%)	***0.008***	15/24 (63%)	***0.03***
8p	17	3/27 (11%)	10/39 (26%)	*0.21*	4/24 (17%)	*0.54*
9p	51	7/28 (25%)	23/39 (59%)	***0.007***	21/23 (91%)	***< 0.0001***
11q	22	4/28 (14%)	5/39 (13%)	*1*	13/23 (57%)	***0.0004***
13q	13	1/28 (4%)	4/37 (11%)	*0.38*	8/23 (25%)	***0.04***
17p	35	3/28 (12%)	17/39 (44%)	***0.006***	15/24 (63%)	*0.20*
High-risk LOH patterns						
>1 arm lost	49	5/28 (18%)	22/39 (56%)	***0.002***	22/24 (92%)	***0.004***
&/or 9p plus others	43	5/26 (19%)	17/37 (46%)	***0.04***	21/24 (88%)	***0.001***

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

VI.4. FV and LOH

As shown in Table 8, FVL lesions showed a consistent increase in LOH frequencies in all categories of comparison, and the increase was significant on individual arm for 9p (73% vs. 31%, $P = 0.0002$) and 11q (33% vs. 11%, $P = 0.03$), and significant

for both high-risk LOH patterns – for multiple loss (64% in FVL vs. 37% in FVR lesions, $P = 0.02$), and for LOH at 3p and/or 9p plus any loss on other arms (62% vs. 29%, $P = 0.004$).

Table 8 Fluorescence visualization alterations and loss of heterozygosity (LOH)

	All	FVR	FVL	*P* value
# of lesions	91	35	56	
LOH on chromosome arm				
3p	26	7/34 (21%)	19/53 (36%)	*0.15*
4q	27	9/33 (27%)	18/53 (34%)	*0.63*
8p	17	8/35 (23%)	9/55 (16%)	*0.58*
9p	51	11/35 (31%)	40/55 (73%)	***0.0002***
11q	22	4/35 (11%)	18/55 (33%)	***0.03***
13q	13	3/34(9%)	10/54 (19%)	*0.36*
17p	35	10/35 (29%)	25/56 (45%)	*0.18*
High-risk LOH patterns				
>1 arm lost	49	13 (37%)	36 (64%)	***0.02***
3p &/or 9p plus others	43	10/34 (29%)	33/53 (62%)	***0.004***

VI.5. FV and outcome (progression into invasive cancer)

Within the mean follow-up time of 29 ± 11 months, 5 of the 66 patients had a lesion progress to invasive SCC. The average time to develop into SCC was 34 ± 14 months.

To test whether FV had value in identifying OPLs at risk of progression into invasive SCC, the relationship between FV and outcome was examined in Table 9. All 5 progressing cases were from FVL lesions, whereas none of the patients with FVR lesions had invasive SCC. However, the difference between two groups was not statistically significant.

Table 9 Fluorescence visualization alterations and progression

	All	FVR	FVL	*P* value
# of patients	66	27	39	
No progression to invasive SCC	61/69 (92%)	27/27 (100%)	34/39 (87%)	*0.07*
Progress to invasive SCC	5/69 (8%)	0/27 (0%)	5/39 (13%)	

VI.5.1. Histology and outcome

In addition, the relationship between histology and outcome was also examined (Table 10). The results showed that increased degree of dysplasia was accompanied by increased frequency of cancer progression. None of the non-dysplastic lesions, 4% (1/28) of the low-grade dysplasia, and 22% (4/18) the high-grade dysplasia cases progressed into SCC ($P = 0.02$).

Table 10 Histology and progression

	All	Non-dysplastic lesions	Low-grade dysplasia	High-grade dysplasia	*P* value
# of cases	66	20	28	18	
No progression to invasive SCC	61	20 (100%)	27 (96%)	14 (78%)	***0.02***
Progress to invasive SCC	5	0 (0%)	1 (4%)	4 (22%)	

VI.5.2. LOH and outcome

Table 11 examined the relationship between LOH patterns and progression into invasive SCC. Progressing lesions showed a consistently higher LOH frequency in all categories of comparison, and the increase was significant with individual arms at 9p (48% in non-progression lesions vs. 100% in progressing to SCC $P = 0.05$), and 11q (19% in non-progression lesions vs. 75% in progressing to SCC $P = 0.05$), and significant for both high-risk LOH patterns – for multiple losses (48% in non-progression lesions vs. 100% in progressing to SCC $P = 0.05$), and for LOH at 3p &/or 9p plus any other arm (43% vs.100%, $P = 0.02$).

Table 11 LOH and progression

	All	No progression to invasive SCC	Progressed to invasive SCC	*P* value
# of lesions	66	61	5	
LOH on chromosome arm				
3p	20	17/58 (29%)	3/5 (60%)	*0.32*
4q	18	15/58 (26%)	3/5 (60%)	*0.14*
8p	11	10/60 (17%)	1/5 (20%)	*1*
9p	34	29/60 (48%)	5/5 (100%)	***0.05***
11q	17	14/61 (19%)	3/4 (75%)	***0.05***
13q	11	9/60 (15%)	2/5(40%)	*0.20*
17p	18	22/61 (36%)	3/5 (60%)	*0.36*
High-risk LOH patterns				
>1 arm lost	34	29/61 (48%)	5/5 (100%)	***0.05***
p &/or 9p plus others	30	25/58 (43%)	5/5 (100%)	***0.02***

VII. DISCUSSION

The prognosis of oral SCC remains one of the worst among major human cancers, with a 5-year survival rate around 50%. This dismal prognosis has not changed over the last 3~4 decades. Late diagnosis of the disease contributes significantly to the poor prognosis. For example, the 5-year survival rate for oral SCC diagnosised in the late stages is around 20 to 30%, compared to 80% 5-year survival rate for early stage SCC. Improving our ability to identify the high-risk oral premalignant lesions and early oral cancer is crucial for improving the prognosis of oral SCC.

The current gold standard for the identification of high-risk OPLs and early oral cancer is histopathology. The gold standard is conclusive for the diagnosis of oral cancer including early cancer, and is also excellent for identifying high-risk OPLs when the degree of dysplasia in an OPL is pronounced (severe dysplasia or *CIS*), but poor in cancer prediction when the degree of dysplasia is not pronounced. With rapid development in molecular technologies, intensive research has been focused on the development of molecular markers that would help to differentiate high-risk OPLs with minimal or no dysplasia from those low-risk OPLs with minimal or no dysplasia. For example, recent research including that of this research team have also shown molecular analysis of biopsy samples using microsatellite analysis for LOH could markedly improve our ability to predict the cancer risk of OPLs. However, both the gold standard, histology, and molecular analysis are currently dependent upon tissue biopsies submitted by clinicians.

Without tissue biopsies or with wrong biopsies (biopsy taken from wrong area of a lesion), histology and most of the current molecular assays are useless.

At present the decision of when and where to biopsy is based on the clinical risk factors including site, size and clinical appearance of the lesions, smoking habit and head and neck cancer history of the patients. The clinical criteria, however, are far from adequate for the identification of high-risk OPLs because of two major problems: one, OPLs could be easily mistaken for reactive or inflammatory lesions of no malignant potential (no biopsy is needed most of the time for these lesions); and too many OPLs and early cancer are not clinically visible, or apparent. Visual aids are urgently needed, and have becoming another recent research focus.

Our research team has recently developed FV, a hand-held simple device that could directly visualize tissue autofluorescence (direct fluorescence visualization, or direct FV). This thesis describes early results from an ongoing prospective study of 91 premalignant/preinvasive oral lesions in which FV status was evaluated for the ability to facilitate the identification of lesions at high risk of progression into invasive oral SCC. This capacity was judged by examining histological risk factors (presence of dysplasia and high-grade of dysplasia), molecular risk factors (high-risk LOH patterns) and by outcome (progression into invasive SCC). The preliminary results suggested that FVL lesions have a higher cancer risk than FVR lesions.

The following is a summation of the evidence from this thesis in support of the use of FV as a visual tool to help clinicians identify high-risk oral lesions and as a risk predictor for OPLs with histological, molecular and outcome data.

VII.1.FV losses increased in frequency with histological progression

Histopathology is the current gold standard in judging the malignant risk of OPLs and it has been successfully applied for decades in different organs and tissues such as upper aerodigestive tract, lung, breast, uterine cervix and skin. It is therefore very important that any new visual tool should be examined for its ability to identify histologically high cancer risk lesions, particularly those with high-grade dysplasia (severe dysplasia/*CIS*) since these lesions are well recognized to have a high chance of progression into invasive cancer. In the following paragraphs, the ability of FV as a tool in identifying dysplastic lesion will be discussed.

Loss of FV was associated significantly with the presence of dysplasia. While 43% (16 of 28) of non-dysplastic lesions showed FVL, 70% (44 of 63) of dysplastic lesions showed FVL ($P = 0.02$; Table 4). Similarly loss of FV was also associated significantly with the degree of dysplasia. High-grade dysplasia showed markedly higher frequency of FVL than that of low-grade dysplasia: 100% (24/24) of high-grade dysplasia showed FVL as compared to 51% (20/39) of low-grade lesions ($P < 0.0001$, Table 5). These results suggest that FVL happens in the majority of the dysplastic lesions and all of the high-grade lesions and support the use of FV as a visual aid to identify high-risk oral lesions.

It should be noted that Table 4 in the usual sense was showing the sensitivity and specificity of FV. Since 70% of the dysplastic lesions showed FVL, and 43% of non-dysplastic lesions showed FVR, one could say that the sensitivity of FV in identifying dysplastic lesions was 70% (100% in identifying high-grade lesions, Table 5), and specificity was 43%. This would indicate a low specificity. The thesis is not regarding

this as the specificity because the sample source of the study. The non-dysplastic lesions used in this study were mostly 'white patches' clinically (indication of possible premalignancy), and were from patients at risk of cancer development – patients with oral dysplasia/oral cancer or a history of these lesions. Such leukoplkia have a very high progression risk even with little or no dysplasia. In other words, the non-dysplastic lesions used in this study are not the same as nondysplastic lesions taken from general population because they do have increased cancer risk, hence we could not judge the specificity of the device here. Currently, we have begun to evaluate the device in patients in community settings and the true specificity should be available when we have studied enough samples from general population.

VII.2.FV revealed lesions with high-risk molecular patterns

As discussed before, microsatellite analysis for LOH has been found to be a powerful tool in differentiating high-risk OPLs from histologically similarly low-risk OPLs. Recent studies from a number of laboratories have shown that high-risk OPLs are characterized by elevated LOH frequencies, and microsatellite analysis for LOH could be powerful tool in differentiating high-risk OPLs with minimal or low-grade dysplasia from those low-risk OPLs with minimal or low-grade dysplasia (Califano *et al.* 1996; Mao *et al.* 1996; Partridge *et al.* 1999; Rosin *et al.* 2000). OPLs with multiple LOH, particularly those with LOH at 3p &/or 9p plus loss at other arms are particularly at risk for cancer progression (a 33-fold increase in relative cancer risk). Consequently another important step in validating new visual tool is to examine the ability of the new visual tool to identify molecularly high-risk lesions.

Consistent with data from the previous studies (Rosin *et al.* 2000), this thesis showed that increased LOH frequencies and high-risk LOH patterns were associated with the presence of dysplasia and increasing degrees of dysplasia. Similarly FVL lesions showed increased LOH frequency in almost all categories of comparison, and the increase was significant for the high-risk molecular patterns: 64% of FVL lesions showed multiple losses as compared to 37% of FVR lesions ($P = 0.02$, Table 8); 62% of FVL lesions showed LOH at 3p and/or 9p plus loss at any other chromosome arm as compared to 29% of FVR lesions ($P = 0.004$, Table 8). Again the molecular study results from this thesis support the use of FV as a visual tool to identify OPLs at risk of cancer progression.

VII.3.FV status correlated with outcome

The ultimate validation of any risk predictor, markers or visual tools is outcome. This is very hard to do, especially for prospective studies because of the difficulty and rarity of longitudinal studies. Because our research team is running one of the largest longitudinal studies for oral premalignant lesions globally, this thesis was able to produce data on this difficult front. However, because this is a newly developed device, and the research team has not been using the device for very long, the follow-up information is limited. Of the 66 lesions studied here, the mean follow-up time was 29 ± 11 months. Within this follow-up period, 5 of the 66 patients had invasive oral SCC developed from their oral lesions. The average time to develop into SCC was 33 ± 14 months.

The study results showed that presence and increasing degree of dysplasia is associated with risk of the lesions progressing into invasive SCC: none of the 20 lesions

without dysplasia had progressed into invasive SCC; only 1 of 28 (4%) low-grade dysplasia progressed into invasive SCC; whereas 4 of 18 (22%) of high-grade dysplasia progressed into invasive cancer (P =0.02, Table 10). Although an abundance of literature from retrospective studies of the oral cavity has indicated the strong association between cancer progression and presence and degree of dysplasia, few prospective studies are available. The data here provide some rare information regarding the progression rate and speed. Longer follow-up will produce further valuable data.

The study results also confirmed both the retrospective and prospective studies from this lab that presence of high-risk molecular patterns is essential for progression of OPLs: All of the 5 lesions that progressed into cancer demonstrated multiple losses and LOH at 3p and/or 9p plus loss at any other chromosome arm. However, similarly to previous studies, a significant proportion of lesions that have not progressed into invasive SCC also contained high-risk LOH patterns. If it is possible with longer follow-up time, some of the lesions with the high risk molecular pattern will develop into invasive SCC. On the other hand, in addition to the essential presence of high-risk LOH patterns, there may be other mechanisms (yet to be revealed) playing critical roles in facilitating the progression of lesions with such high-risk LOH pattern to cancer.

When the relationship between FV and outcome was examined, the study data showed that all 5 cases that progressed into cancer were lesions with FVL. Although the difference was not significant, 0% (0/27) of FVR lesions progressed into SCC vs. 13% (5/39) of FVL lesions (P = 0.07, Table 9), this is probably is a result of small sample numbers and limited follow-up time. The fact that all of the progressing cases were FVL indicates the high sensitivity of FV in identifying lesions at risk for progression.

All the data support the use of FV as a first-stage screening tool to identify lesions at risk for progression. To be followed by histological and LOH analysis based on the biopsy as the second and third stage. This multi-stage screening system, we can be expected to improve the prognosis of oral cancer.

VII.4. Limitations of the study and future plans

Due to the time restraint for a MSc thesis, to date, this longitudinal project could only study 91 biopsies in 66 patients, which consequently makes the power of study as only 0.65 (GPOWER was used to calculate the power of this study and estimate the increased sample size of future study, in which the alpha value of 0.05 and the effect size of 0.5 were used. The analysis was two-sided.). Therefore, a larger number of samples are needed to minimize the errors of statistic analysis.

Because of the limitation in sample size, some analyses were not possible for this study, such as analysing primary OPLs (those from patients without a history of oral cancer) and 'secondary' OPLs (those from patients with a history of oral cancer) separately.

The 66 lesions used in this study were part of an ongoing longitudinal study in the Dysplasia Clinic at BCCA. As discussed before, these patients had either a history of primary oral dysplasia or a history of oral SCC, consequently the non-dysplasia used in this study was not representing "true" non-premalignant control lesions.

Finally the average follow-up time in my thesis is only 29 months, whereas carcinogenesis occurs over a long period, perhaps 10 to 15 years. More high-risk cases may progress into cancer, so the time of follow-up these patients need to be increased.

VII.5.Summary

The results of this study showed that FV could identify high-risk OPLs as judged by histology (high-grade dysplasia), by molecular profiles (high-risk LOH patterns), and by outcome (increased cancer progression). The device is already being marketed in the United States for use in the clinician's office. Currently our research team is verifying the results by increasing sample sizes, adding different sample groups (i.e., samples from normal population), increasing follow-up time, and identifying confounding factors. If the study results are confirmed, and the device is also tested in different research centres with similar results, it will have important clinical implications, supporting its use by clinicians as an adjunct tool to facilitate the identification of the high-risk OPLs, including clinically occult lesions. Success in early identification and management of these lesions should lead to improvements in the prognosis and management of oral SCC.

VIII. APPENDX RAW DATA FILE

Bx#	Tube ID	Histological grade[1]	SCC Progression	Date of Birthday	Date of Biopsy	Date of Last Visit	Follow -up time[2] (mon)	FV status	Age[3]	Sex	ETHICITY	EVER-SMOKER	LOH at 3p[4]	LOH at 4q[4]	LOH at 8p[4]	LOH at 9p[4]	LOH at 11q[4]	LOH at 13q[4]	LOHat 17p[4]
02UD-1366	964D1-1	D1	No	Sep-1945	May-2002	Jun-2002	2	R	56	F	WHITE	Yes	L	L	R	L	R	L	L
02UD-1290	911D1-1	D1	No	Apr-1957	Apr-2002	Jul-2002	3	R	44	M	WHITE	Yes	R	L	L	R	R	R	L
02UD-1811	963D1-1	D1	No	Jul-1955	Jun-2002	Sep-2002	4	R	46	M	WHITE	Yes	L	L	R	L	L	L	R
02UD-2508	703N4-4	NoDYS	No	May-1917	Sep-2002	Mar-2003	7	R	84	M	WHITE	Yes	R	R	R	R	R	R	R
UD03-2140	882H1-1	NoDYS	No	May-1943	Jul-2003	Mar-2004	9	R	59	M	WHITE	Yes	R	R	R	R	R	R	R
UD03-1062	53D5-1	D2	No	Oct-1913	Apr-2003	Mar-2004	11	R	89	F	WHITE	Never	R	NI	R	R	R	R	L
02UD-3055	587D6-1	D2	No	Dec-1930	Oct-2002	Dec-2003	14	R	71	M	WHITE	Yes	L	L	L	L	R	R	L
UD04-192	918D1-1	D1	No	Oct-1938	Jan-2004	Apr-2005	15	R	65	M	WHITE	Yes	R	R	R	L	R	R	R
UD03-2579	981D1-2	D1	No	Jun-1944	Sep-2003	Jun-2005	21	R	58	F	N/A	Yes	R	R	R	L	R	L	R
UD03-2245	521D2-2	D1	No	Mar-1959	Aug-2003	Aug-2005	25	R	43	M	WHITE	Yes	R	R	L	R	R	R	R
UD03-3032	884D1-1	NoDYS	No	Dec-1917	Nov-2003	Dec-2005	25	R	85	M	WHITE	Yes	R	R	R	L	L	R	L
02UD-3340	618D3-3	NoDYS	No	Nov-1923	Nov-2002	Jan-2005	27	R	78	F	WHITE	Yes	R	R	R	L	R	R	R
UD04-339	993D1-1	D1	No	Jan-1950	Feb-2004	May-2006	28	R	53	F	WHITE	Never	R	L	R	R	R	R	L
UD03-3300	910H1-1	NoDYS	No	Jan-1956	Dec-2003	Apr-2006	29	R	46	M	WHITE	Yes	L	R	L	R	R	R	R
UD03-3300	910H1-2	NoDYS	No	Jan-1956	Dec-2003	Apr-2006	29	R	46	M	WHITE	Yes	R	R	R	R	R	R	R

Bx#	Tube ID	Histological grade[1]	SCC Progression	Date of Birthday	Date of Biopsy	Date of Last Visit	Follow -up time[2] (mon)	FV status	Age[3]	Sex	ETHICITY	EVER-SMOKER	LOH at 3p[4]	LOH at 4q[4]	LOH at 8p[4]	LOH at 9p[4]	LOH at 11q[4]	LOH at 13q[4]	LOHat 17p[4]
UD03-3169	986D1-1	D1	No	Mar-1957	Nov-2003	Mar-2006	29	R	45	M	ASIAN	Yes	R	R	R	R	L	R	R
UD03-1209	875D1-1	D1	No	Jun-1941	Apr-2003	Oct-2005	30	R	61	F	ASIAN	Never	NI	L	L	R	L	R	R
UD03-3334	897D1-2	D2	No	Jun-1958	Dec-2003	May-2006	30	R	44	M	WHITE	Yes	R	L	R	L	R	NI	L
UD03-2167	700H2-1	NoDYS	No	Oct-1960	Jul-2003	Feb-2006	31	R	42	F	Dominican Republic	Never	R	R	R	R	R	R	R
UD03-2167	700H2-2	NoDYS	No	Oct-1960	Jul-2003	Feb-2006	31	R	42	F	Dominican Republic	Never	R	R	R	R	R	R	R
UD03-2459	877D2-1	D2	No	Dec-1947	Sep-2003	Mar-2006	31	R	55	M	WHITE	Never	R	L	L	L	R	R	R
UD03-947	468H2-1	NoDYS	No	Jan-1949	Apr-2003	Jan-2006	34	R	53	F	WHITE	Never	R	R	R	R	R	R	L
UD03-2180	530NE5-2	NoDYS	No	Sep-1953	Aug-2003	May-2006	34	R	49	F	WHITE	Yes	R	R	R	R	R	R	R
UD03-946	705D4-3	D2	No	Apr-1934	Apr-2003	Mar-2006	35	R	68	M	WHITE	Yes	R	NI	R	L	R	R	R
03UD-205	823D4-1	D2	No	Jun-1958	Jan-2003	Dec-2005	35	R	44	M	ASIAN	Yes	R	R	R	R	R	R	L
03UD-361	877D1-1	D2	No	Dec-1947	Feb-2003	Mar-2006	38	R	55	M	WHITE	Never	L	L	L	L	R	R	R
03UD-225	879H1-1	NoDYS	No	Aug-1955	Jan-2003	Mar-2006	38	R	47	F	WHITE	Yes	R	R	R	R	R	R	L
03UD-225	879H1-2	NoDYS	No	Aug-1955	Jan-2003	Mar-2006	38	R	47	F	WHITE	Yes	R	R	R	R	R	R	R
03UD-119	880H1-2	NoDYS	No	May-1917	Jan-2003	May-2006	40	R	85	F	WHITE	Yes	R	R	R	R	R	R	R
02UD-2421	785D2-1	D1	No	Jan-1950	Aug-2002	Mar-2006	43	R	51	M	WHITE	Yes	R	R	R	R	R	R	R
02S-16632	359D10-1	D1	No	May-1934	May-2002	Apr-2006	48	R	67	M	WHITE	Yes	R	R	R	R	R	R	R
02UD-2990	876H1-3	NoDYS	Lost	Jan-1935	Oct-2002	N/A	N/A	R	66	M	WHITE	N/A	L	R	R	R	R	R	R
02UD-2990	876NE1-4	NoDYS	Lost	Jan-1935	Oct-2002	N/A	N/A	R	66	M	WHITE	N/A	R	R	R	R	R	R	R
02S-16794	883H1-1	NoDYS	Lost	May-1951	May-2002	N/A	N/A	R	50	M	WHITE	Yes	R	R	R	R	R	R	R
UD03-2508	885VH1-2	D2	Lost	May-1949	Sep-2003	N/A	N/A	R	53	M	N/A	N/A	L	R	L	R	R	R	R
UD05-3459	992H2-4	NoDYS	No	Jan-1961	Nov-2005	Jan-2006	3	L	43	M	WHITE	Yes	R	R	R	R	R	R	R

Bx#	Tube ID	Histological grade[1]	SCC Progression	Date of Birthday	Date of Biopsy	Date of Last Visit	Follow-up time[2] (mon)	FV status	Age[3]	Sex	ETHICITY	EVER-SMOKER	LOH at 3p[4]	LOH at 4q[4]	LOH at 8p[4]	LOH at 9p[4]	LOH at 11q[4]	LOH at 13q[4]	LOHat 17p[4]
UD04-509	915D1-1	D3	No	May-1935	Feb-2004	Aug-2004	6	L	68	F	WHITE	Never	R	R	R	L	L	R	L
02UD-2508	703D4-1	*CIS*	No	May-1917	Sep-2002	Mar-2003	7	L	84	M	WHITE	Yes	R	L	R	L	R	R	L
02UD-2508	703D4-2	D1	No	May-1917	Sep-2002	Mar-2003	7	L	84	M	WHITE	Yes	R	R	R	L	R	R	L
02UD-2508	703D4-3	*CIS*	No	May-1917	Sep-2002	Mar-2003	7	L	84	M	WHITE	Yes	R	L	R	L	R	R	L
UD04-776	916D1-1	D3	Yes	Nov-1958	Mar-2004	Feb-2005	11	L	45	F	ASIAN	Never	R	L	R	L	L	R	L
UC05-648	958D1-3	D3	No	Jun-1950	Mar-2005	Jan-2006	11	L	54	F	WHITE	Never	R	R	R	L	R	R	R
02UD-2767	835VH1-1	D3	No	Jan-1931	Sep-2002	Sep-2003	13	L	70	F	WHITE	Yes	L	L	R	L	R	L	R
UD04-2290	867D3-1	D3	No	Jul-1942	Aug-2004	Dec-2005	17	L	61	M	GERMAN / ENGLISH / POLYNESIAN / FIJIAN	Yes	L	R	R	L	R	R	L
UD04-1956	933D1-1	D2	No	Apr-1943	Jul-2004	Dec-2005	17	L	60	M	WHITE	Yes	L	R	R	L	L	R	L
UD04-1551	929D1-1	D3	No	Jul-1931	May-2004	Mar-2006	22	L	72	F	WHITE	Yes	R	R	R	R	R	R	R
UD03-2527	630D4-1	D1	No	Oct-1933	Sep-2003	Jul-2005	23	L	69	M	WHITE	Never	R	R	R	R	R	R	R
UD04-774	407D4-1	D3	No	Apr-1962	Mar-2004	Mar-2006	25	L	41	M	WHITE	Never	L	R	R	L	L	R	R
UD03-2245	521D2-1	D1	No	Mar-1959	Aug-2003	Aug-2005	25	L	43	M	WHITE	Yes	R	R	L	R	R	R	R
UD03-2481	875D2-1	NoDYS	No	Jun-1941	Sep-2003	Oct-2005	25	L	61	F	ASIAN	Never	NI	R	R	R	R	R	R
UD03-2481	875D2-2	NoDYS	No	Jun-1941	Sep-2003	Oct-2005	25	L	61	F	ASIAN	Never	NI	R	R	R	R	R	R
UD03-3032	884D1-2	D1	No	Dec-1917	Nov-2003	Dec-2005	25	L	85	M	WHITE	Yes	R	R	R	L	R	R	R
UD03-3332	603D3-1	D3	No	Sep-1952	Dec-2003	Feb-2006	27	L	50	F	EAST OR SOUTH-EAST ASIAN	Yes	L	L	L	L	L	L	L
02UD-3340	618D3-1	D2	No	Nov-1923	Nov-2002	Jan-2005	27	L	78	F	WHITE	Yes	L	R	R	L	R	R	R

Bx#	Tube ID	Histological grade[1]	SCC Progression	Date of Birthday	Date of Biopsy	Date of Last Visit	Follow -up time[2] (mon)	FV status	Age[3]	Sex	ETHICITY	EVER-SMOKER	LOH at 3p[4]	LOH at 4q[4]	LOH at 8p[4]	LOH at 9p[4]	LOH at 11q[4]	LOH at 13q[4]	LOHat 17p[4]
UD03-3031	864D3-1	D2	No	Jun-1952	Nov-2003	Jan-2006	27	L	50	F	WHITE	Never	R	R	R	L	R	R	L
UD03-3031	864D3-2	D3	No	Jun-1952	Nov-2003	Jan-2006	27	L	50	F	WHITE	Never	R	L	R	L	R	R	L
UD04-413	932D1-1	D2	No	Jan-1945	Feb-2004	May-2006	27	L	58	M	WHITE	Yes	L	L	L	L	R	L	L
UD04-150	844D2-1	D3	No	Apr-1946	Jan-2004	May-2006	28	L	57	M	ASIAN	Yes	L	L	L	L	L	L	L
UD03-3029	886D2-1	*CIS*	No	Jun-1934	Nov-2003	Mar-2006	29	L	68	M	ASIAN	Yes	L	L	R	NI	L	L	L
UD03-3100	845D2-1	D1	Yes	Apr-1926	Nov-2003	May-2006	30	L	76	F	WHITE	Never	R	R	L	L	R	R	L
UD03-1209	875D1-2	D2	No	Jun-1941	Apr-2003	Oct-2005	30	L	61	F	ASIAN	Never	NI	R	R	R	R	R	L
UD03-2891	982D1-1	D2	No	Feb-1931	Oct-2003	Apr-2006	30	L	71	F	WHITE	Never	R	R	R	L	L	R	R
UD03-2165	617VH7-1	NoDYS	No	Oct-1948	Jul-2003	Feb-2006	31	L	54	F	WHITE	Yes	R	R	R	L	R	R	R
UD03-999	701D2-1	D2	No	Sep-1954	Apr-2003	Oct-2005	31	L	48	F	WHITE	Yes	L	R	R	L	R	R	L
UD03-2180	530D5-1	D1	No	Sep-1953	Aug-2003	May-2006	34	L	49	F	WHITE	Yes	R	R	R	R	R	R	R
UD03-483	867D2-1	D3	No	Jul-1942	Feb-2003	Dec-2005	34	L	60	M	GERMAN / ENGLISH / POLYNESIAN / FIJIAN	Yes	L	L	R	L	L	L	L
02UD-3254	666H4-1	NoDYS	No	Mar-1920	Nov-2002	Oct-2005	35	L	81	M	WHITE	Never	R	R	R	L	L	R	R
UD03-946	705D4-1	D2	No	Apr-1934	Apr-2003	Mar-2006	35	L	68	M	WHITE	Yes	R	NI	R	L	R	R	R
UD03-946	705D4-2	D2	No	Apr-1934	Apr-2003	Mar-2006	35	L	68	M	WHITE	Yes	L	NI	R	L	R	R	R
UD03-448	864D2-1	D3	No	Jun-1952	Feb-2003	Jan-2006	36	L	50	F	WHITE	Never	R	L	R	R	R	R	L
UD03-448	864D2-2	D1	No	Jun-1952	Feb-2003	Jan-2006	36	L	50	F	WHITE	Never	R	R	R	R	R	R	L
03UD-225	879H1-3	NoDYS	No	Aug-1955	Jan-2003	Mar-2006	38	L	47	F	WHITE	Yes	R	R	R	R	R	R	R
UD03-515	621T2-1	*CIS*	No	Jan-1940	Feb-2003	May-2006	40	L	62	F	WHITE	Never	L	L	R	L	R	L	L
03UD-119	880H1-1	NoDYS	No	May-1917	Jan-2003	May-2006	40	L	85	F	WHITE	Yes	R	R	R	R	R	R	R

Bx#	Tube ID	Histological grade[1]	SCC Progression	Date of Birthday	Date of Biopsy	Date of Last Visit	Follow-up time[2] (mon)	FV status	Age[3]	Sex	ETHICITY	EVER-SMOKER	LOH at 3p[4]	LOH at 4q[4]	LOH at 8p[4]	LOH at 9p[4]	LOH at 11q[4]	LOH at 13q[4]	LOHat 17p[4]
02UD-2866	210D13-1	D3	No	Oct-1958	Oct-2002	Feb-2006	41	L	43	F	WHITE	Never	L	R	R	L	R	R	L
02UD-3201	872H1-1	NoDYS	No	Jun-1954	Nov-2002	Apr-2006	41	L	47	M	WHITE	Yes	R	R	NI	R	R	R	R
02UD-2701	833D1-1	*CIS*	No	Jun-1942	Sep-2002	Mar-2006	42	L	59	M	WHITE	Yes	L	L	R	L	L	R	R
02UD-2701	833D1-2	*CIS*	Yes	Jun-1942	Sep-2002	Mar-2006	42	L	59	M	WHITE	Yes	L	L	R	L	L	L	R
02UD-2701	833D1-3	*CIS*	Yes	Jun-1942	Sep-2002	Mar-2006	42	L	59	M	WHITE	Yes	L	L	R	L	L	L	R
02UD-2702	834H1-1	D2	No	May-1957	Sep-2002	May-2006	44	L	44	M	WHITE	Never	R	NI	R	L	R	R	L
02UD-2503	845D1-2	D3	Yes	Apr-1926	Sep-2002	May-2006	44	L	75	F	WHITE	Never	L	R	R	L	NI	R	L
02UD-2606	871D1-1	D1	No	Mar-1933	Sep-2002	May-2006	44	L	68	F	ASIAN	Never	R	R	R	R	R	R	R
02UD-2703	987VH/D1-1	NoDYS	No	Sep-1943	Sep-2002	May-2006	44	L	58	F	WHITE	Yes	R	R	R	L	L	L	R
02UD-2674	797D5-1	D1	Lost	Apr-1962	Jul-2002	N/A	N/A	L	39	F	WHITE	Never	R	L	R	L	R	R	L
UD03-2942	869D2-1	*CIS*	Lost	Apr-1943	Oct-2003	N/A	N/A	L	59	F	WHITE	Yes	R	R	L	L	L	R	R
UD03-2942	869D2-2	*CIS*	Lost	Apr-1943	Oct-2003	N/A	N/A	L	59	F	WHITE	Yes	R	L	L	L	L	R	R
UD03-2942	869D2-3	D2	Lost	Apr-1943	Oct-2003	N/A	N/A	L	59	F	WHITE	Yes	R	R	R	L	R	NI	R
UD03-2942	869D2-4	D3	Lost	Apr-1943	Oct-2003	N/A	N/A	L	59	F	WHITE	Yes	L	R	R	L	L	NI	L
02UD-2990	876H1-1	NoDYS	Lost	Jan-1935	Oct-2002	N/A	N/A	L	66	M	WHITE	N/A	R	L	L	L	L	R	R
02UD-2990	876H1-2	NoDYS	Lost	Jan-1935	Oct-2002	N/A	N/A	L	66	M	WHITE	N/A	R	R	L	R	R	R	R
UD03-3033	909H1-1	NoDYS	Lost	Dec-1962	Nov-2003	N/A	N/A	L	40	M	ASIAN	Yes	R	R	R	L	R	R	R

[1]Histological grade: NoDYS: Oral lesions without dysplasia (reactive hyperplasia or inflammation); D1: Oral lesions with mild dysplasia; D2: Oral lesions with moderate dysplasia; D3: Oral lesions with severe dysplasia; *CIS*: Carcinoma *in situ*.

[2]Follow-up time: Months from diagnosis to last clinical visit.

[3]Age: Years of age at diagnosis

[4]LOH evaluation: for each loci, L, loss of heterozygosity; R, retention (no loss of heterozygosity); NI, non-informative.

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