FLUORESCENCE *IN-SITU* HYBRIDIZATION OF *3P14.2* AND *9P21* TUMOUR SUPPRESSOR GENES AS A TOOL FOR IDENTIFYING ABNORMAL GENETIC PATTERNS ASSOCIATED WITH INCREASED ORAL CANCER RISK

by

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PROJECT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ENVIRONMENTAL TOXICOLOGY

In the Department of
Biological Sciences

SIMON FRASER UNIVERSITY

2007

APPROVAL

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Degree: Master of Environmental Toxicology

Title of Project:

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ABSTRACT

Oral cancer is typically diagnosed in late stages, resulting in poor prognosis and high mortality. Early detection is essential to improving survival. Fluorescence *in-situ* hybridization of oral mucosa cells detected abnormal genetic patterns in *3p14.2* and *9p21* in a population characterized with a higher than average risk for developing oral cancer. We hypothesized that these patients would contain a greater proportion of individuals with higher frequencies of detectable genetic abnormalities at *3p14.2* and *9p21* and their respective chromosomes, compared to a representative average-risk population. The data showed that while centromere deletions were more common at *3p* in high-risk patients, *3p14.2* mutations were only significant when grouped with centromere alterations. In contrast, gene deletions were common at *9p21* with significant differences in *9p* centromere amplification. These results suggest that *3p* chromosome loss, *9p21* gene loss and *9p* chromosome amplification are potential indicators of elevated oral cancer risk.

Keywords: chromosome instability, FISH, *FHIT*, oral cancer, cancer screening

DEDICATION

To my mother and father for showing me that determination and hard work is the foundation to a fulfilling and successful life.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Miriam Rosin. Her dedication and devotion to her research is an inspiration to any scientist who has had the privilege of working with her. I thank Dr. Margo Moore for her help throughout the duration of my project as well as her involvement in related preceding projects, which played a vital role in shaping my current research project. I thank Dr. Lewei Zhang for her expertise in the clinical aspect of my project. I also thank Dr. Catherine Poh for leading the Portland Community Clinic study and providing her insightful perspective on my research project. I would also like to extend my thanks to my colleagues at the Cancer Prevention Lab at B.C. Cancer Agency, particularly Alisa Kami and Brenda Currie for their involvement at the Portland Clinic and Jennifer Choo for her help in the B.C. Cancer laboratory. I also thank Dr. Sean Sikorski at the Portland Community Clinic and his staff for their support during the course of my research.

I would also like to thank Dr. Christopher Kennedy. His advice and guidance over the years has been instrumental in shaping my academic career and I am very grateful for his help.

Lastly, I would like to thank Kelli Bergh and Srinivas Sura, my friends and peers in the MET program with whom we have shared all the joys, frustrations and achievements of graduate and personal life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

170K19	*E.coli* strain containing plasmid sequence for *3p14.2*
91A15	*E.coli* strain containing plasmid sequence for CEP3
ADAMTS	A disintegrin-like and metalloproteinase with thrombospondin protein
ADC	Abacus Dental Clinic
BAP	Benzo[a]pyrene
BAC	Bacterial artifical chromosome
CDK4	Cyclin-dependent kinase 4
CIS	Carcinoma *in-situ*
CEP3	Chromosome enumeration probe for chromosome 3
CEP9	Chromosome enumeration probe for chromosome 9
CYP	Cytochrome P_{450}
DAPI II	4,6-diamidine-2-phenylindole
DTES	Downtown East-side
FHIT	Fragile histidine triad
FISH	Fluorescence *in-situ* hybridization
GI	Gastrointestinal
HCA	Heterocyclic amine
HPV	Human papilloma virus
LOH	Loss of heterozygosity
MRP	Multidrug resistant protein
NNK	N-nitrosamine-4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
OPL	Oral premalignant lesion
OSCC	Oral squamous cell carcinoma
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCC	Portland Community Clinic
PhIP	2-amino-1-methyl-6-phenylimidazo-(4,5-b) pyridine

QHQ	Quinone-hydroquinone complex
RB	Retinoblastoma
SNK	Student-Newman-Keuls test
SSC	Sodium-chloride-sodium citratein buffer
TSG	Tumour suppressor gene
VELScope	Visually Enhanced Lesion scope
WHO	World Health Organization

1. INTRODUCTION

1.1 Overview of Oral Cancer

Oral cancer is the sixth most common cancer, accounting for approximately 275,000 new cases annually worldwide. This disease represents 3% of all cancers in Canada and in the United States (4.9 in males and 1.9 in females per 100,000) (Franceschi *et al.*, 2000) with a rate of 11 in 100,000 (Parkin *et al.*, 2005). In developing countries, particularly in South-East Asia, this rate increases dramatically to 40% to 50% of all diagnosed cancers. Melanesian countries have the highest incidence of oral cancer with a rate of 31.5 in 100,000 for men and 20.2 in 100,000 for women (Parkin *et al.*, 2005). This elevated rate is due to increased tobacco and alcohol exposure, the two major risk factors for oral cancer. An additional contributing factor is the frequent practice in developing countries of chewing tobacco-containing components, which increases local exposure of the oral cavity to carcinogens. In many South-East Asian countries, heavy tobacco and alcohol use is integrated into the culture, making the practice common and widely acceptable.

Despite technological advances in cancer treatment, the Canadian and American 5-year survival rate for oral cancer has remained unchanged since 1960 at approximately 50% (Franceschi *et al.*, 1993). In the United States, the 5-year survival rate varies between 34% to 58%; African-Americans tend to have lower survival rates compared to Caucasian populations possibly due to lack of accessible health care . In developing countries, where access to sufficient health care is limited, the 5-year survival rate plummets to only 20% (Jemal *et al.*, 2005). The long-term prognosis of oral cancer

improves significantly when malignant lesions are discovered at earlier stages. Advances in treatment methods such as surgery, radiotherapy and chemotherapy are not always aimed at curing the disease. The use of these treatments at late stages of tumour development is aimed at prolonging or improving quality of life rather than eliminating the cancer. The lack of effective early detection methods is a key factor in the dismal survival rate for oral cancer. This is compounded by patient delays in reporting oral premalignant lesions (Lopez-Jornet and Camacho-Alonso, 2006), and also professional delays; specifically, the referral of a patient by a dentist to an oral cancer specialist (Onizawa *et al.*, 2003). Another cause for the low survival rate is the recurrence of malignancies after treatment of the primary cancer, which occurs in approximately 20-30% of all oral cancer cases (Kowalski *et al.*, 1993). In these cases, treatment methods such as surgery fail to entirely eliminate cancerous tissue allowing the development of new tumours, which are genetically related to the original growth (Doll *et al.*, 2004).

These poor survival statistics have fuelled research in developing new methods to detect early signs and risk factors of the disease in hopes of preventing rather than treating the cancer.

This thesis will be focusing on early diagnosis in a high-risk population. In the following sections, I will first review the etiologies for oral cancer, carcinogen metabolism, and current clinicopathological diagnosis of oral cancer. Then, I will discuss the current problem in early diagnosis and the need to reach high-risk populations and to develop new molecular tools. Finally, I will review the molecular markers *3p* and *9p* and their use in the diagnostic tool fluorescence *in-situ* hybridization (FISH).

1.2 Etiology of Oral Squamous Cell Carcinoma (OSCC)

The development of oral cancer results from the possible interactions of a variety of environmental factors as well as an individual's inherent genetic susceptibility. Tobacco exposure, excessive alcohol use, diets low in fibre, fruits and vegetables and exposure to human papilloma virus are the primary known causes of oral cancer, each of which may be preventable through education. Non-preventable factors include those associated with inherent genetic susceptibility such as reduced expression of detoxification enzymes and/or the presence of genetic polymorphisms that reduce the activity of phase I and II enzymes such as glutathione-S-transferase (Frandsen *et al.*). The large number and complex interaction of factors leading to OSCC makes risk quantification very difficult. For this study, only preventable causes were examined in the populations surveyed and they are described in more detail below.

1.2.1 Tobacco

Tobacco use, both smoking and smokeless, is the leading cause of cancer and of overall mortality worldwide responsible for an estimated 4 million deaths worldwide annually (Greenlee *et al.*, 2001). The public perceives smoking as the primary cause of lung cancer; however, research also implicates its involvement in cancers in many other sites of the body including oral, nasal, pharyngeal, pancreas, bladder and cervical cancers (DeMarini, 2004). Tobacco exposure accounts for an estimated 30% of all diagnosed cancers in developed countries (Vineis *et al.*, 2004).

The carcinogenicity of tobacco condensates has been established for over 50 years (Izzotti *et al.*, 2001). Over 4000 chemicals in tobacco smoke and its condensates have been identified, many of which are genotoxic (Hoffmann and Hoffmann, 1997). Tobacco

smoke condensates are genotoxic in almost every animal system in which they have been tested. These condensates have been shown to induce a wide variety of genetic aberrations including: sister chromatic exchanges, micronuclei formation, aneuploidy, DNA adduct formation, DNA strand breaks and elevated levels of translocation in somatic cells (Channarayappa *et al.*, 1992; DeMarini, 2004; Fielding *et al.*, 1989; Leanderson and Tagesson, 1992; Veltel and Hoheneder, 1996). In addition, the sperm of smokers has increased rates of aneuploidy, DNA adducts, strand breaks and oxidative damage indicating that the harmful effects extend to germ cells (Rubes *et al.*, 1998; Shi *et al.*, 2001).

Carcinogenic compounds in tobacco include polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, formaldehyde and metals such as chromium and arsenic. Over 68 tobacco-related carcinogens have been evaluated by the International Agency for Research in Cancer in various animal models with PAHs and N-nitrosamines having the greatest carcinogenic potential. Most are also lipophilic in nature, allowing them to diffuse through cellular and nuclear membranes to interact with DNA or intracellular receptors (Smith *et al.*, 2003).

Tobacco chewing is also an important risk factor for oral cancer, exposing tissues to carcinogens found naturally in cigarettes without pyrolytic reactions. N-nitrosonornicotine (NNN) was the first organic carcinogen to be isolated from non-pyrolysed tobacco and is found in its highest concentrations in fermented and finely cut chewing tobacco (Fischer *et al.*, 1990). Since then, seven NNN carcinogens have been identified in chewing tobacco (Hoffmann *et al.*, 1994). Use of chewing tobacco has been linked to not only oral cancer but also to the development of OPLs. For example, in one

study, 196 of 423 non-smoking, smokeless tobacco users developed an OPL compared with 7 of 493 people who did not use any tobacco products (Grady *et al.*, 1990). NNN is the by-product of bacterial nitrosation of nicotine, which is also formed by the reaction of salivary nitrates, a natural antibacterial, and nornicotine. NNN levels increased by 44% in chewing tobacco mixed with saliva, levels 1,000 times greater than in mainstream tobacco smoke (Hecht *et al.*, 1974). NNNs are precarcinogens, requiring metabolic activation by CYP 2A6 enzymes found in the saliva to become biologically active carcinogens (Kamataki *et al.*, 2002).

The most potent carcinogenic compounds from tobacco are not inherently carcinogenic and are known as precarcinogens. Precarcinogens must undergo metabolic reactions to become active carcinogens, a process known as bioactivation. The most researched PAH, benzo[a]pyrene, and other PAHs require bioactivation by Phase I cytochrome P_{450} enzymes to become DNA-reactive species. Bioactivated PAHs covalently bind to DNA and form adducts mainly with adenine and guanine nucleotide bases (Pryor, 1997). Unless removed by DNA-repair mechanisms, these genetic lesions cause mutations during cell proliferation leading to the initiation of cancer as well as other degenerative diseases. Figure 1 shows one of many pathways by which BAP can be bioactivated to form DNA-reactive species. BAP is composed of five fused benzene rings and has no inherent carcinogenic or mutagenic effects due to the lack of reactive functional groups. However, the organic and lipophilic nature of BAP allows it to easily diffuse through cellular membranes. A groove in the molecular structure of BAP known as the "bay region", allows the molecule to associate with the major and minor grooves of

the DNA double helix (Stowers and Anderson, 1985). However, without any functional groups on BAP, no reaction takes place with DNA.

In a series of cytochrome P_{450} catalysed reactions, BAP is metabolised to form increasingly water soluble products, including the ultimate carcinogen (+)benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide. The carcinogenic molecule is still highly lipophilic, despite the addition of alcohol and epoxide functional groups. Furthermore, the bay region now contains a highly reactive epoxide functional group, which binds covalently to guanine nucleotides and form DNA adducts. If left unrepaired, DNA adducts may affect DNA synthesis in the S-phase of the cell cycle leading to multiple types of damage including DNA strand breaks or mismatched nucleotides (Leanderson and Tagesson, 1992). If a mutation in a critical gene region is not repaired, it can lead to either activation of an oncogene or deactivation of a tumour suppressor gene causing a gradual deregulation of the cell cycle. Multiple mutational events of this kind lead to aberrant cells that lose control over normal growth, potentially leading to cancer.

Alternatively, BAP may also undergo other pathways of metabolism that result in cytotoxic, but not mutagenic metabolites such as ortho-quinone and benzo[a]pyrene-4,5-dihydrodiol.

The tobacco-specific N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is also believed to play a major role in tobacco-induced carcinogenesis. NNK also requires bioactivation from CYP1A2, CYP2A6 and CYP3A4 to become DNA-reactive, which also forms DNA adducts (Hecht, 1996).

Cigarette smoke also contains free radicals that induce oxidative damage to cellular membranes and DNA. A quinone-hydroquinone (QHQ) complex contained in the

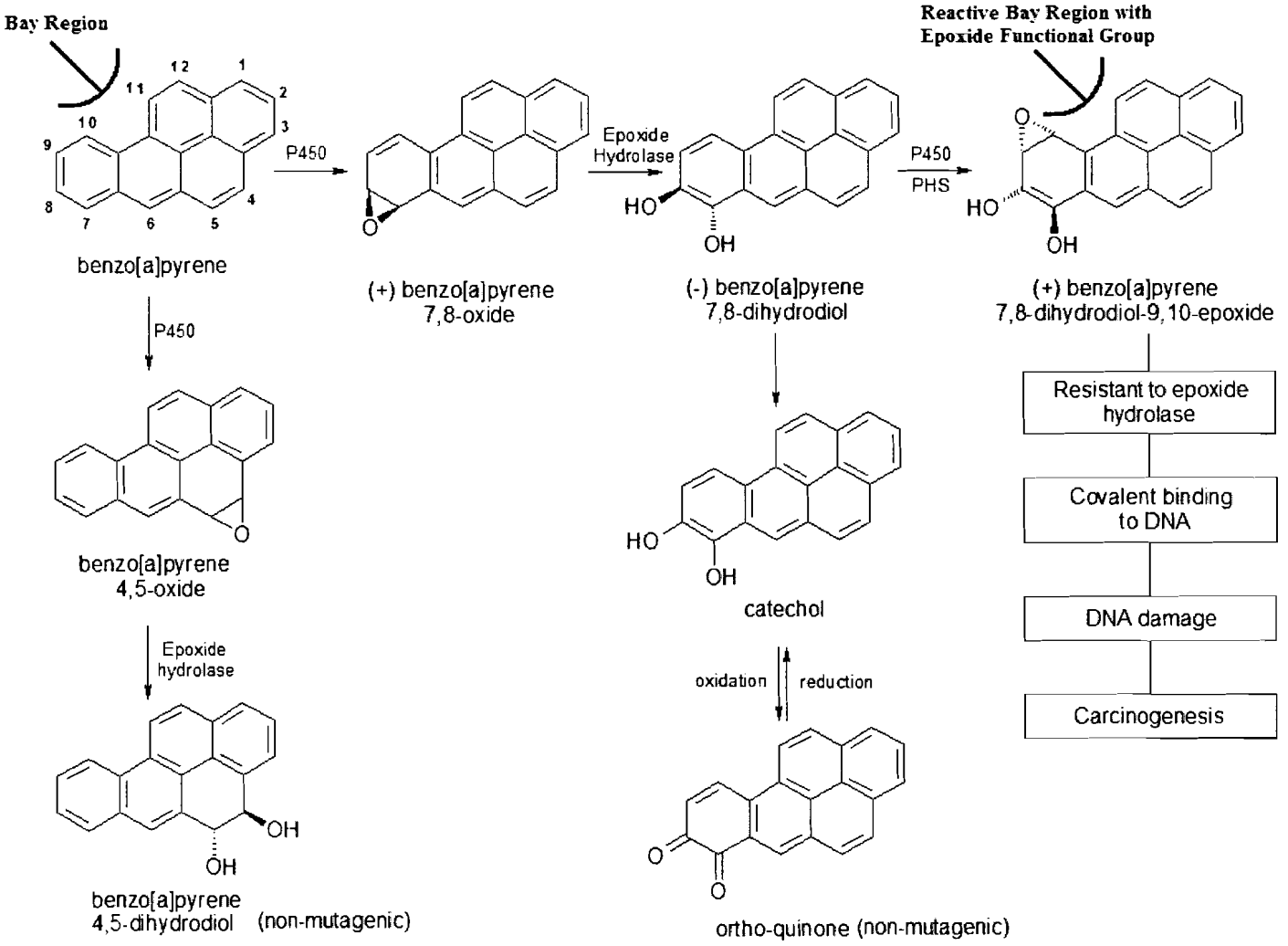
tar component of cigarettes is postulated to be the main source of free radical inducing compounds. Nitric oxide is also found in high levels in smoke particulates. QHQ studies show it forms a redox reaction in cells to produce hydrogen peroxide and hydroxyl radicals that cause DNA nicking and single-stranded DNA breaks in rodent and human cells. Nitric oxides in the gaseous phase act in a similar fashion to generate peroxynitrite. The presence of amplified DNA damage in cultured cells exposed to whole cigarette smoke compared with fractionated components suggests that both QHQ and nitric oxide gas may act synergistically with other tar components (Pryor, 1997).

Chewing tobacco habits consist of the placement and retention of finely ground or powdered tobacco between the gum and cheek. Its practice is associated with multiple cancers of the mouth, primarily in tissues that come into direct contact with the tobacco powder such as the tongue, cheek and lower gum. The popularity of chewing tobacco in the United States was at its peak during 1940-1980 among rural women and women working in textile factories as an alternative to smoking tobacco, which was banned for fire safety purposes (Blot and Fraumeni, 1977). Subsequently, its practice in developed countries has diminished significantly, but tobacco-related cancers continued to increase with the popularity of smoking tobacco.

Another form of tobacco chewing is as a component of betel quid use, a common practice in South-East Asia. Betel quids are mixtures of tobacco, areca nut and lime that are rolled in a betel leaf to be chewed or held in place underneath the tongue. The effects of betel quid are believed to be potentiated by the damaging effects of lime on cellular tissues, which initiates tissue repair and cellular replication, acting as a promoting agent (Lin *et al.*, 2002). In addition to betel quids, many Asian countries also use other

smokeless tobacco products such as gutkha and pan masala, the latter containing crushed betel nut, tobacco and a variety of spices and flavouring (Nair *et al.*, 2004), which are marketed as candies to children. Betel quid-induced chromosomal changes are similar to that of tobacco smoking and has been strongly implicated in the development of OSCC (Chang *et al.*, 2002).

Figure 1 Metabolic bioactivation of benzo[a]pyrene to the ultimate carcinogen (+)benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide



Adapted and modified from Klaassen (2001)

1.2.2 Alcohol

Alcohol is an important risk factor in the development of oral cancer. Its consumption has been established as an independent factor associated with an estimated 18% and 77% of head and neck cancers in non-smoking and smoking drinkers, respectively (Lee *et al.*, 2007). The relative risk of oral cancer from drinking up to 29 alcoholic beverages per week in the absence of smoking is 1.6 relative to non-smoking non-drinkers. It increases up to 5.8 when consumption exceeds 29 alcoholic beverages a week (Blot *et al.*, 1988).

Separation of alcohol and tobacco effects on OSCC is difficult because of the long-standing correlation between heavy drinking and tobacco smoking (Wydner *et al.*, 1957). Alcohol consumption in conjunction with tobacco potentiates OSCC relative risk from 35 to 107.9-fold (compared to individuals who do not smoke or regularly consume alcohol) depending on alcohol and tobacco consumption, duration and age (Blot *et al.*, 1988; Lewin *et al.*, 1998).

There are two widely accepted hypotheses for alcohol potentiation of tobacco-induced OSCC. The first suggests that alcohol exposure of oral mucosa cells alters activity of enzymes involved in metabolism of tobacco carcinogens. PAHs in tobacco smoke require bioactivation by phase I cytochrome enzymes and specific cytochrome enzymes can be induced as a result of alcohol exposure in oral mucosa cells, particularly CYP 2E1 (Ronis *et al.*, 1993). Other carcinogens such as NNK require expression of CYP 2E1 and 2C7 (Yamazaki *et al.*, 1992). Although NNK is known to be bioactivated by CYP 1A1, CYP 2A6 and CYP 3A4, its activation by CYP 2E1 has not been demonstrated conclusively although it is believed to play some role in cancer risk

(Badger *et al.*, 2003). The CYP 2C class of cytochromes are responsible for metabolism of steroid hormones (Gonzalez, 1989). Since PAHs such as BAP structurally resemble the cholesterol component of steroid hormones, metabolism of BAP by CYP 2C7 is hypothesized to bioactivate the reaction product by adding reactive functional groups to the bay region of the molecule, with the subsequent formation of DNA adducts (Hakkak *et al.*, 1996; Pronko *et al.*, 2002). The potential role of alcohol in CYP 2C activity is not known. Aryl hydrocarbon hydroxylase also known as CYP 1A1, is another group of cytochrome P_{450} enzymes that bioactivate PAH. However, its induction results from PAH exposure rather than alcohol, leading to a positive feedback response (Kushinsky and Louis, 1976). The induction accelerates the rate of bioactivation of these compounds thereby increasing the rate of DNA adduct formation to exposed cells.

The second hypothesis proposes that metabolic breakdown of ethanol by CYP 2E1 forms acetaldehyde, a carcinogenic and mutagenic compound that has been shown to bind to DNA and proteins as well as to generate reactive oxygen species (Poschl *et al.*, 2004).

In addition to the two aforementioned hypotheses, other modes of action of alcohol have been identified as potentially playing a role in carcinogenesis. Short-chained alcohols such as ethanol are amphipathic and act as a solvent for membrane-integrated lipids. This effectively solublizes or disrupts membrane lipids and cholesterol molecules making cellular membranes more permeable to carcinogens and other molecules (Watson and Reitz, 1993). In addition, alcohol can also act as a solvent for lipophilic carcinogens leading to higher aqueous phase concentrations (Reitz, 1993). The combination of elevated bioactivation rate and exposure potential is believed to be the mechanism by

which alcohol potentiates the carcinogenic effect of tobacco smoke (Badger *et al.*, 2003), particularly in the floor of the mouth where residual alcohol and tobacco condensates pool.

Although studies do not prove conclusively whether the alcohol content in beer, wine or spirits differ with respect to the degree of potentiation, the frequency of consumption is correlated to OSCC risk (Barra *et al.*, 1990; Veer *et al.*, 1989). Subsequently, this risk increases when drinking patterns are addictive rather than social. Evidence also suggests similar mechanisms of risk from alcohol-based mouthwashes with 40% and 60% elevated risks in men and women, respectively in one study of leukoplakia that involved 2000 individuals. Oral leukoplakia are clinical alterations that are associated with the presence of OPLs and cancer, described in more detail in section 1.4.1. Leukoplakia risk was elevated among users of mouthwashes with higher alcohol content, some of which contain up to 30% denatured alcohols (Winn *et al.*, 1991).

1.2.3 Dietary Intake

The role of diet in carcinogenesis is extremely difficult to establish, though this is not due to lack of research in this field. A large quantity of literature exists on the role of diet in a variety of cancers, but the accuracy of quantifying food intake over time is poor at best. For example, in this thesis, an initial attempt was made to quantify dietary intake in the examined populations. Inconsistencies in individual recollection of past dietary habits, variation in interpretation of questions and highly unstable dietary habits made accurate quantification virtually impossible. A large volume of literature suggests that intake of adequate quantities of fruits, vegetables and fibre are protective against cancers (Goodwin and Brodwick, 1995; Patterson *et al.*, 1995; Terry *et al.*, 2001). For some

cancers such as breast (van Gils *et al.*, 2005) and prostate (Cohen *et al.*, 2000), other studies have shown diet plays little to no significant protective role. Inconsistency of data collection and variations within and between studies may be factors leading to this uncertainty. Most studies on head and neck cancer, which includes oral, naso-pharyngeal and throat have shown that fruit and vegetables are protective (Block *et al.*, 1992). Other sites such as liver, pancreas, breast and liver have shown highly conflicting results.

Fruit consumption is shown to have an inverse relationship with oral cancer (De Stefani *et al.*, 2005; McLaughlin *et al.*, 1988). Fruits and vegetables are a good source of antioxidants (Enwonwu and Meeks, 1995). These antioxidants, such as vitamins A, E, C and beta carotene are free radical scavengers which neutralize reactive oxygen species, preventing them from damaging DNA. Other components of fruits and vegetables may interfere with pre-carcinogen activation, prevent carcinogen binding to DNA, inhibit chromosome aberrations and suppress actions of cancer promoters (Enwonwu and Meeks, 1995). The protective qualities of fruits is shown through studies which found that OSCC patients and smokers had lower blood-serum levels of antioxidants as well as increased levels of membrane lipid peroxidation (Cowan *et al.*, 1999; Subapriya *et al.*, 2003). A related study shows that fruit intake was related to increased blood-serum antioxidant levels and a reduction in many forms of cancer (Winn, 1995).

The association of other food components is less well-established. For example, meats and starch-based foods cooked at high temperature have been shown to be associated with production of carcinogens. The role of such carcinogens in oral cancer is not established. Pyrolysed meats contain compounds known as heterocyclic amines (HCAs), which are a group of pre-carcinogenic chemicals that require bioactivation prior

to adduct formation with DNA (Frandsen *et al.*, 1992). 2-amino-1-methyl-6-phenylimidazo-(4,5-b) pyridine (PhIP) is one such HCA that is produced as a result of the reaction of creatine with the amino acid L-phenylalanine at high temperatures. Studies have shown higher temperature cooking methods such as fire barbeques or deep frying produces the highest concentrations of HCA in meats (Sinha *et al.*, 2000). Due to their lipophilic nature, they can diffuse across cell and nuclear membranes in the oral mucosa.

Starch-based foods cooked at high temperatures also produce mutagenic substances, particularly acrylamide, which has been found in french fries and boxed cereals (Tareke *et al.*, 2002). It is also used in some water filtration devices and may leave trace concentrations in treated water (Bolto, 1995). Similar to HCAs, higher concentrations are produced with greater cooking temperatures (Tareke *et al.*, 2002). Nevertheless, concentrations of acrylamide in food are relatively low and the most recent large-scale research has suggested that acrylamide can be disregarded as a significant factor in any form of cancer due to the average net dietary intake (Erdreich and Friedman, 2004; Ruden, 2004). However, genetic mutations are cumulative over time and damage from chronic low doses of acrylamide and HCAs may significantly elevate an individual's lifetime risk of cancer (Ruden, 2004).

1.2.4 Human Papilloma Virus

Human papilloma virus (HPV) is an important factor in many types of cancers including the cervix, other sites in the genital anal region and, head and neck. There are over 130 strains of HPV, many of which are oncogenic. HPV 16, 18 and 31 are the primary oncogenic strains. HPV-DNA was detected in approximately 19% of diagnosed oral cancers in a Montreal-based study that examined samples for the presence of HPV

strains 16, 18 and 31 (Pintos *et al.*, 2007). In contrast, in cervical cancer, HPV-DNA is detected in approximately 95% of diagnosed cases, illustrating the importance and association of HPV with that cancer. HPV-DNA from strains 16 and 18 are the most common oncogenic strains and account for up to 80% of all HPV infections (Giovannelli *et al.*, 2002) and 74% of OSCC patients with HPV-DNA have either HPV 16 and/or 18 infection (Zhang *et al.*, 2004).

HPV infects epithelial cells and inserts genes for oncoproteins E6 and E7 into the host genome (See Figure 6). When expressed, the E6 oncoprotein binds and inhibits the function of the *p53* protein. *p53* binds to over 100 cellular receptors. One of its key functions is to act as an inducer of programmed cell death or apoptosis (Webster *et al.*, 2000). Apoptosis occurs naturally during cell turnover or in response to cellular or genetic damage. Binding of *p53* by E6 protein prevents apoptosis from occurring, extending the cell's lifespan and the potential to pass any acquired mutations to daughter cells through mitosis.

pRB protein is a cell cycle regulator, preventing transition from the growth phase (G_1) to DNA synthesis phase (S) in the cell cycle by binding to the transcription factor E2F1 which promotes cell replication. The HPV E7 oncoprotein binds and inactivates *pRB* protein, eliminating the ability to stop cell replication at the G_1-checkpoint of the cell cycle (Weinberg, 1995). Once a cell passes the G_1-checkpoint into the S-phase, it must continue the process of mitosis.

Recently a series of large phase III clinical trials of HPV vaccines have begun, which are aimed at ascertaining the efficacy of the two vaccines, Garasil from Merck, and Ceravix from GlaxoSmithKline in the prevention of HPV infections and in reduction of

development of cervical premalignant lesions and cancer. To date, they have shown 100% efficacy in preventing infection. A fast-track program conducted by the US Food and Drug Administration has approved these vaccines for public use, years ahead of the typical timeline required for drug development and approval. Several US states have approved a voluntary HPV vaccination program for 11- and 12- year-old females, which will be integrated into elementary school vaccination programs (Gostin and DeAngelis, 2007). Because of the high association of HPV with cervical cancers (95%), it is believed that cervical cancer can be virtually eliminated with an efficient vaccine distribution program to the most affected populations. HPV vaccines that target strains associated with oral cancer may eventually be developed to reduce the rates of HPV-linked oral cancers.

1.2.5 Hygiene, Immuno-compromisation, Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS)

Individuals who are immuno-compromised as a result of major surgeries and organ transplants are more susceptible to cancers in general (Penn, 1991). Oral cancer development is most common on the lower lip in these cases (De Visscher *et al.*, 1997). The role of virally-mediated immuno-compromisation, i.e. HIV and AIDS, in cancer development differs depending on the tissue of origin. For example, some studies have shown that HIV and AIDS infected individuals are more susceptible than non-infected individuals, to most cancers such as Hodgkin's Lymphoma, multiple myeloma, leukaemia, lip and lung (Grulich *et al.*, 1999). However, other studies show that OSCC in patients with HIV or AIDS is very rare (Langford *et al.*, 1995), with similar results in cervical cancers (Serraino *et al.*, 1999). Rather than a direct causative agent, the role of

HIV and AIDS in cancer may be due to immuno-compromisation resulting in individuals who are more susceptible to other diseases such as *Candida albicans* and Epstein-Barr virus which are associated with OSCC (Sand *et al.*, 2002).

Poor oral hygiene and oral health are also risk factors for oral cancer (Zheng *et al.*, 1990). Interestingly, one study demonstrated that poor dental hygiene increased acetaldehyde production from ethanol in the saliva (Homann *et al.*, 2001).

1.3 Carcinogen Metabolism and Excretion

The importance of carcinogen metabolism to carcinogenesis has been introduced in sections 1.2.1 and 1.2.2 of this thesis. The following is a more comprehensive explanation.

Metabolic enzymes play critical roles in the biotransformation and elimination of xenobiotics and carcinogens from the body. The primary role of metabolic enzymes is to catalyse reactions that increase the water solubility of the target molecule. Water-soluble molecules are more readily excreted from the body, effectively reducing the exposure duration and, therefore, the toxicity. The toxicity of an endogenous metabolite or a xenobiotic is dependent on both its potency and the exposure duration. Unfortunately, during metabolism, some compounds can become bioactivated, when reactive functional groups are added to normally harmless molecules. This results in a toxic metabolite, which can cause cellular or genetic damage.

The liver is the primary organ for biotransformations where hepatocytes contain the highest concentrations of metabolic enzymes in the body. However, significant

metabolic activity exists in many other tissues including the lungs and the gastrointestinal epithelium (Kaminsky and Fasco, 1991; Shimada *et al.*, 1996).

1.3.1 Phase I Detoxification Mechanisms

Phase I metabolic enzymes catalyse reactions that either add or expose polar functional groups on the target molecule. These include various forms of hydrolysis, reduction and oxidation reactions. Among the oxidative enzymes is the cytochrome P_{450} system. The cytochrome P_{450} system is the most versatile group of enzymes, capable of biotransforming a broad range of xenobiotics. Cytochrome P_{450} (CYP) enzymes are expressed in virtually all cells in the body and can be induced or upregulated in response to exposure to specific xenobiotics. For example, ethanol induces CYP 2E1 expression after ingestion, a CYP which is responsible for the metabolic breakdown of benzenes, nitrosamines and various pharmaceutical drugs (Novak and Woodcroft, 2000). Tobacco smoke and charcoal-broiled beef induce CYP 1A2, which metabolises aromatic amines to carcinogenic metabolites (Meunier *et al.*, 2000). Cytochrome P_{450} induction is temporary and may last from days to weeks depending on the isozyme and inducer.

In some cases, CYP metabolism creates reactive metabolites that are either cytotoxic or mutagenic; a phenomenon known as bioactivation. Benzene, for example, can be oxidized to various quinones and semiquinones that cause hematopoietic toxicities and also leukaemia (Snyder *et al.*, 1993).

1.3.2 Phase II Detoxification Mechanisms

Phase II detoxification is responsible for further increasing the water solubility and/or decreasing the reactivity of lipophilic metabolites and xenobiotics to promote their

excretion through the urine. These enzymes catalyse the conjugation of large polar molecules such as glucuronic acid, sulfonates or amino acids to lipophilic components (Rushmore and Tony Kong, 2002). Compounds with no reactive functional groups such as benzo[a]pyrene require phase I metabolism to add or expose functional groups to provide a reactive site. Since conjugation reactions add non-reactive polar molecules, these conjugates do not promote xenobiotic bioactivation. Other types of Phase II reactions such as acetylation act to cap reactive functional groups with a non-reactive alkane (Klaassen, 2001).

1.3.3 Excretion

The primary excretion pathway of metabolites and xenobiotics is though the urine. Phase I and II metabolites are carried in the blood to the kidney where glomerular filtration occurs. Polar xenobiotics filtered at the glomeruli remain in the tubular lumen to be excreted with urine. Xenobiotics with high lipid/water partition coefficients are reabsorbed by passive diffusion across the tubular membrane. Water, nutrients and salts, due to their polar nature, must be actively reabsorbed into the body (Klaassen, 2001).

Xenobiotics can also be excreted into the urine by active secretion into the tubular lumen. Recent studies have cloned and identified a family of membrane transporters known as multi-drug resistant proteins (MRP) in mammalian cells (Cole *et al.*, 1992), for this purpose. Lipophilic xenobiotics that are reabsorbed into the bloodstream from the kidneys may undergo further metabolism to increase water solubility, become stored in adipose tissue, or undergo excretion through fecal matter. This is accomplished by MRPs in hepatocytes of the liver, which actively transport lipophilic xenobiotics into the bile. Consequently, bile emulsifies the lipophilic substances, forming micelles, which are

released into the small intestine during digestion and excreted with fecal matter. Conditions may favour the reabsorption of xenobiotics from the gastrointestinal (GI) tract. Blood from the GI tract passes through the liver before entering general circulation. Reabsorbed xenobiotics are extracted from the blood by the liver and actively secreted in the bile again until it is excreted, a cycle known as hepatobiliary excretion (Borst *et al.*, 1999; Klaassen, 2001).

1.4 Clinicopathological Identification of Oral Premalignant Lesions (OPL) and Early OSCC

1.4.1 Oral Premalignant Lesions

OSCC is believed to develop from an OPL. An OPL is defined as morphologically abnormal tissue, which if left untreated; has a greater chance of developing into cancer compared to its normal counterpart (World Health Organization Collaborating Centre for Oral Precancerous Lesions, 1978). Frequently, these OPLs are clinically visible and classified as either leukoplakia or erythroplakia, appearing as white or red patches, respectively (Bouquot and Ephros, 1995; Nagao and Warnakulasuriya, 2003). Only a small proportion of OPLs develop into OSCC, posing a dilemma to clinicians and research scientists on how to predict which OPLs ultimately will progress to cancer.

1.4.2 Clinical Features of OPLs: Leukoplakia and Erythroplakia

Leukoplakia, originally named by Enró Schwimmer, is used to describe a white patch on the tongue and is the more common OPL (Schwimmer, 1877). The current definition of leukoplakia is controversial due to its vagueness and lack of usefulness as a

diagnostic description. The World Heath Organization (WHO) defines it as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease". Therefore, other possible causes of the lesion such as the yeast *Candida albicans* (candidiasis) or oral lichen planus, an immune-mediated inflammation, must be excluded before it is diagnosed since there is no definitive characteristic that applies only to leukoplakia.

The rate of malignant transformation varies greatly between studies. Table 1 shows the malignant transformation rate of oral leukoplakia based on studies conducted in various countries and years. These values range from 0.13% to 31.4%.

Table 1 Malignant transformation rate of oral leukoplakia

Author	Country	Number of patients	Malignant transformation rate (%)
(Lee *et al.*, 2000)	U.S.A.	70	31.4
(Schepman *et al.*, 1998)	Netherlands	166	12
(Bouquot *et al.*, 1988)	U.S.A.	463	10.3
(Silverman *et al.*, 1984a)	U.S.A.	257	17.5
(Gupta *et al.*, 1989)	India	360	0.3
(Gupta *et al.*, 1980)	India	410	2.2
(Banoczy, 1977)	Hungary	670	6
(Silverman *et al.*, 1976)	India	4762	0.13
(Roed-Petersen, 1971)	Denmark	331	3.6
(Silverman, 1968)	U.S.A.	117	6
(Pindborg *et al.*, 1968)	Denmark	248	4.4
(Einhorn and Wersall, 1967)	Sweden	782	4
(Leonardelli and Talamazzi, 1950)	Italy	268	19.8
(Sturgis and Lund, 1934)	U.S.A.	143	13

Modified from Bouquot *et al.* (1995), Silverman *et al.* (1984) and (Nakamura, 2002).

The term *erythroplasia* was originally used by the French dermatologist, Louis Auguste Queyrat to describe a red precancerous lesion on the penis (Queyrat, 1911).

Currently, the modified term *erythroplakia* is used to describe similarly red lesions in the oral cavity. Similar to leukoplakia, erythroplakia is defined as "a red patch that cannot be defined clinically or pathologically as any other condition" and excludes inflammatory conditions, which may appear red. Although more rare, erythroplakia is associated with a higher risk of OSCC. In a study of 65 erythroplakia cases, all showed some degree of dysplasia with 51% being OSCC, 40% carcinoma *in-situ* (CIS) or severe dysplasia and 9% mild or moderate dysplasia (Shafer and Waldron, 1975). As with leukoplakia, diagnosis of this condition also relies on eliminating other diseases as possible causes. Erythroplakia can appear identical to inflamed tissue making it exceptionally difficult to diagnose with the naked eye. Diagnosis is typically the result of biopsy tissue samples, which are examined by an experienced pathologist to determine the degree of cellular abnormality and dysplastic change.

Not all OSCC cases are preceded by a clinically visible OPL. A survey in India showed that only 80% of oral cancer cases were preceded by either leukoplakia or erythroplakia (Gupta *et al.*, 1989). Other researchers suggest that the majority of oral cancers arise from clinically normal mucosa, although it is believed that the mucosa is histologically and molecularly abnormal (Cowan *et al.*, 2001).

OPL and OSCC are typically identified in clinical practice in several ways. The most common method is the result of a dental examination. A dentist may notice a lesion in the oral cavity. Usually the patient is referred to a specialist for further examination or for a biopsy.

The second most common means of identification is by the patient, who notifies their family dentist or doctor of the lesion. In these instances, the patient rarely makes

such an effort unless the lesion is either painful or bleeding, strong but unreliable indicators that the lesion has progressed beyond early stages of cancer development (Neville and Day, 2002).

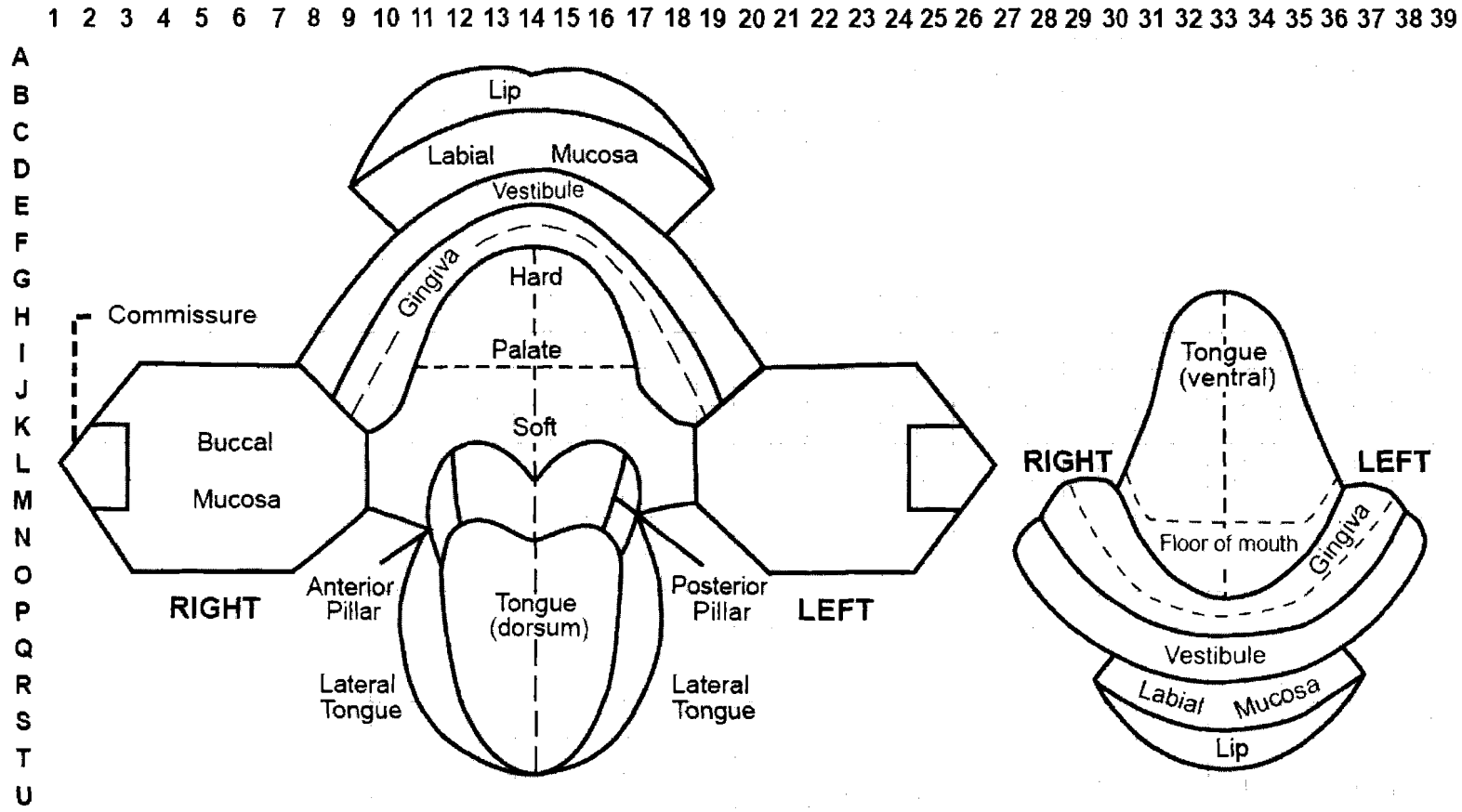
1.4.3 High-Risk Sites of the Oral Cavity for Cancer

Different areas of the oral cavity do not share the same degree of risk for developing OSCC. One of the characteristics for OPLs and OSCC is that they tend to occur in certain oral sites, defined as high-risk sites. The anatomy of the oral cavity is shown in Figure 2. This diagram was used during sample collection in this study to record regions in the mouth or lip with abnormalities in the tissue. Each square on the grid represents a 10mm x 10mm area in the oral cavity. The high-risk locations for oral cancer in Western countries are the ventrolateral tongue and floor of the mouth; whereas the intermediate risk sites are the soft palate, anterior tonsillar pillar tissue and lingual regions of the retromolar area (Reichart and Philipsen, 2005). The remaining areas of the oral cavity are low-risk regions. Approximately 75% to 90% of cancers in Western countries originate in these high-risk areas (Shafer and Waldron, 1975). In contrast, in South Asia, the most common sites for oral cancer are the buccal mucosa, gingival and vestibular mucosa, ventrolateral tongue and the floor of the mouth, due to the habit of chewing tobacco containing betel leaf.

The basis for high-risk at the ventrolateral tongue and the floor of the mouth lies in the fact that these tissues are exposed to greater levels of carcinogenic xenobiotics. While smoking, all of the mucosa is exposed directly to tobacco-related carcinogens for a short period of time. Many of the carcinogens are in particulate phase rather than gaseous phase. Upon contact with the oral tissues, the particulates will either dissolve in or be

suspended in saliva. Due to gravity, chemicals in saliva tend to pool in the lower potions of the oral cavity such as the ventrolateral tongue and the floor of the mouth, effectively prolonging exposure to these carcinogens (Neville and Day, 2002; Wertheimer-Hatch *et al.*, 2000). The same exposure principles may also apply to non-tobacco related carcinogens such as alcohol, heterocyclic amines from meats and acrylamide from starch based foods. On the other hand, for people with the habit of chewing tobacco or tobacco-containing quid, the practice of storing chewed tobacco in the pits of the cheek accounts for the high incidence of cancer of the cheek, gingival and vestibular mucosa.

Figure 2 A schematic of the oral cavity employed during this study to localize the sites of oral lesions.
Each square represents a 10mm x 10mm area.



1.4.4 Histological Features of OPLs and OSCC

After the identification of a potentially high-risk OPL or OSCC either by clinical features or by the site of the lesion, a biopsy is conducted and examined by a pathologist to determine the degree of dysplasia within the tissue. Histology is currently recognized as the gold standard in identifying, predicting and grading an OPL or OSCC (Mehrotra *et al.*, 2006).

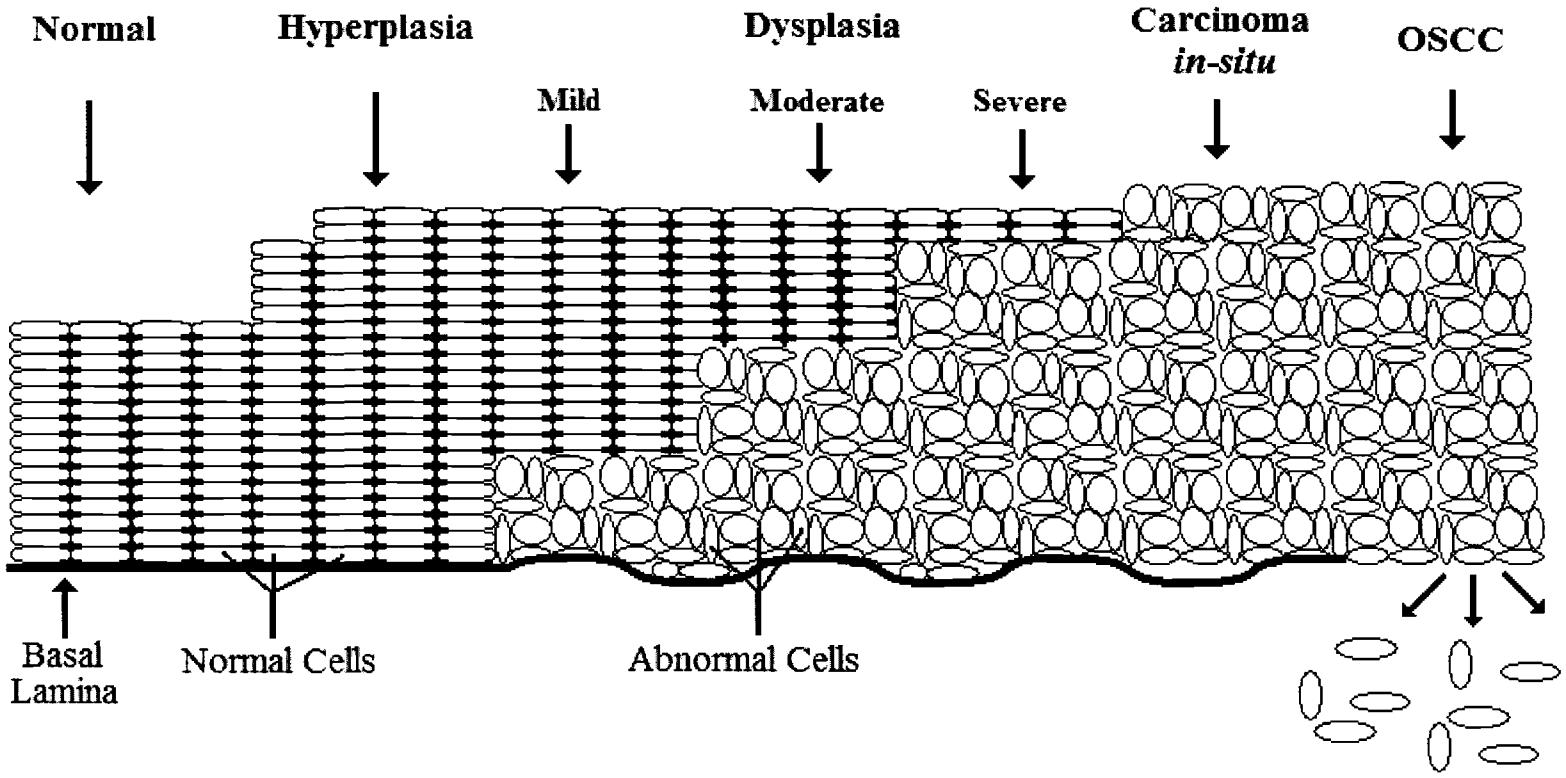
Dysplasia is the abnormal appearance of cells and architecture of the epithelium that are indicative of steps leading to cancer. Certain cellular characteristics must be present, as outlined by the World Health Organization (WHO), for lesions to be diagnosed as dysplastic (World Health Organization Collaborating Centre for Oral Precancerous Lesions, 1978). These criteria include, but are not limited to:

1. Presence of more than one layer of basaloid cells
2. Irregular stratification of tissue layers
3. Presence of mitotic elements in the upper half of the epithelium
4. Loss of basal cell polarity
5. Reduction in cellular cohesion of adjacent cells
6. Increased nuclei size or nuclear/cytoplasmic ratio
7. Altered nuclear shape
8. Nuclear hyperchromatism
9. Nuclear and cellular pleomorphism

Additionally, dysplasia is also ranked as mild, moderate or severe based on the degree of abnormality and the proportion of the cell layers involved in dysplastic changes. Severely ranked OPLs with dysplasia can be classified as carcinoma *in-situ* (CIS), synonymous with high-risk dysplasia. A model of the sequence of histological changes in carcinogenesis is shown in Figure 3. Normal cells become hyperplastic, a state showing increased proliferation but maintenance of normal tissue structure. As an OPL forms, the tissue becomes increasingly dysplastic (Califano *et al.*, 1996; Todd *et al.*, 1997). In mild dysplasia, cellular and structural changes in the tissue structure are seen in one third of the epithelium. In moderate dysplasia, these changes spread to two-thirds of the epithelium whereas severe dysplasia spreads beyond two thirds of the epithelium. The dysplastic lesion may eventually become CIS, where dysplastic changes are seen through the entire epithelial layer but localized within the epithelium. Malignant transformation into OSCC occurs when the cells penetrate the basal lamina, allowing the cancer to spread to the underlying tissues, circulatory and/or lymphatic systems.

Figure 3 Histological progression model

Illustration showing various degrees of dysplasia leading to OSCC



1.5 Problems in Identifying High-Risk OPLs and Early OSCC

The oral cavity is easy to examine and the risk factors for oral cancer are known. Oral cancer typically takes decades to develop, with known premalignant changes that precede invasive cancer. It is therefore surprising that early detection rates remain poor, even in developed countries. The difficulty in detection, diagnosis and elimination of oral cancer is due to a number of factors including those associated with the patient, clinicopathological features and with treatment.

1.5.1 Patient Problems

For patients, many premalignant lesions and early cancers can go unnoticed because most of these lesions are asymptomatic or small; in addition, many patients with oral cancer are in the lower socio-economic class and do not have regular oral examinations. Patients are also less likely to report lesions to a clinician unless they bleed or become painful, features characteristic of high-risk lesions. Painless, non-bleeding lesions are more likely to go unreported; however, these lesions are lower risk (Reichart and Philipsen, 2005). When people with such lesions do seek medical or dental help, they are frequently diagnosed incorrectly, due to the lack of definitive diagnosis criteria in early lesions.

1.5.2 Marginalized Poor and the Downtown East-side

This study involves patient populations in two sites: a control group from a regular dental clinic with average risk for oral cancer. The Abacus Dental Clinic (ADC), located in Mission, British Columbia and a high-risk group, from the Portland Community Clinic (PCC), in the Downtown East-side (DTES) of Vancouver, British

Columbia. The DTES area, defined within the postal code prefix V6A and V6B, is the poorest region in Canada based on average annual income (Statistics Canada, 2001). It represents the heart of Canada's largest drug-using population. The cumulative effects of elevated smoking habit, excessive alcohol consumption, HPV infection, HIV-induced immuno-compromisation, poor diet, a high incidence of disease, illicit drug usage and poor living conditions are factors related to the classification of the DTES population as high-risk for OSCC. The following sections describe characteristics of individuals in these two groups.

Figure 4 is a geographical layout of the Lower Mainland in B.C. depicting government designated community health areas (CHA). CHA#1 and CHA#2 represent Downtown Vancouver and CHA#2 contains the DTES region. CHA#2 has an estimated population of 51,886, with the DTES region defined as an area of 10 square blocks within CHA#2 with a postal code prefix of V6A or V6B. The DTES has an estimated population of 16,275 people in 2004 (Buxton, 2005).

Relative to the provincial rate, only CHA#1 and CHA#2 have a higher relative risk of alcohol-related deaths. The latter is defined as deaths related to alcohol use associated with overdose and health complications and excluding motor-vehicle accidents. Such deaths accounted for 46 and 83 deaths in CHA#1 and CHA#2 in 2003 out of 1789 deaths province-wide. The CHA#2 region accounted for 498 reported alcohol-related deaths between 1998-2002 with the highest mortality rate in B.C. In comparison, the ADC is primarily composed of middle-income patients where drinking patterns are more social.

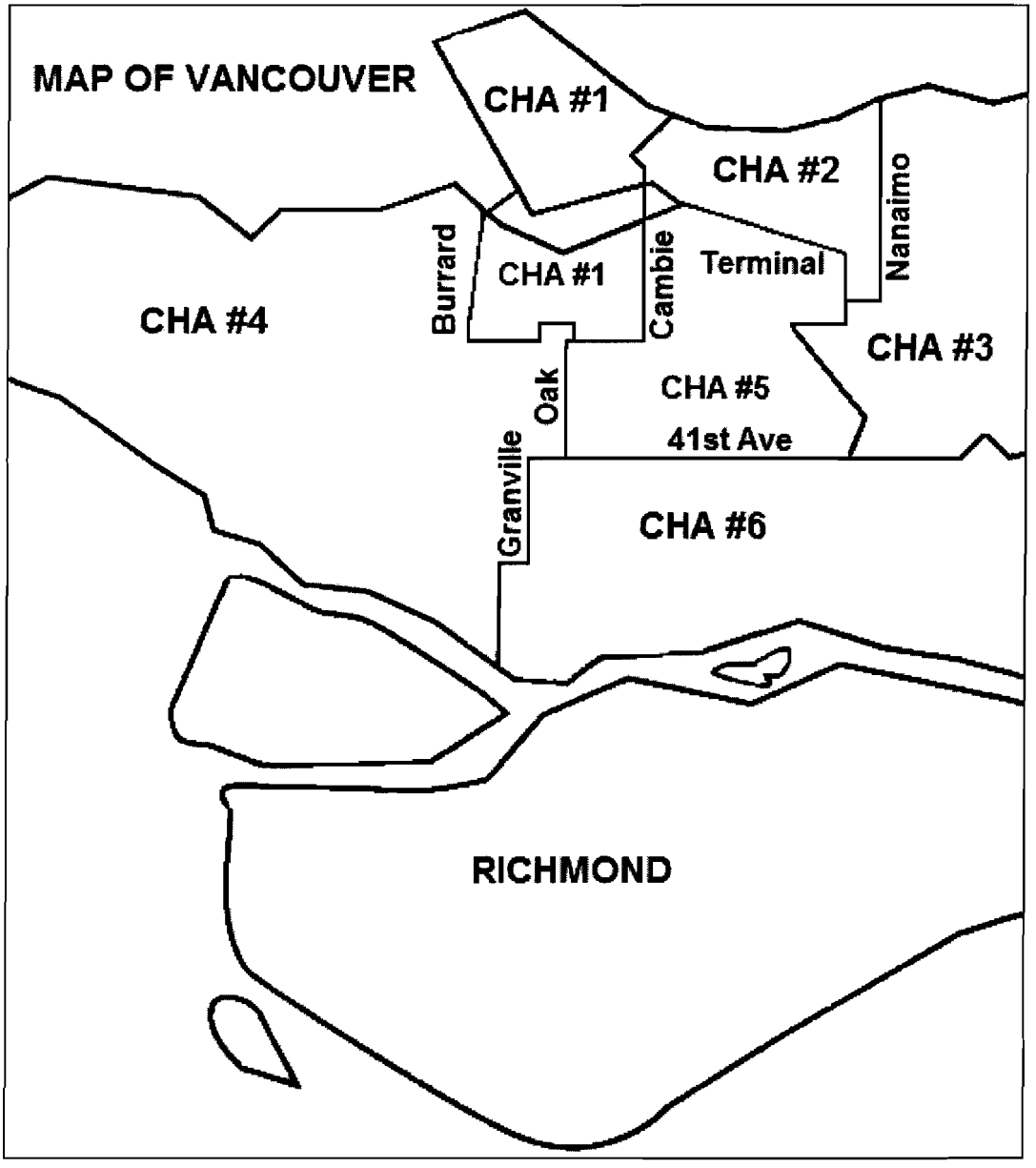


Figure 4 Government designated Community Health Areas (CHA) in the Lower Mainland of British Columbia

Vancouver, British Columbia, Canada is divided into 5 Community Health Areas that are outlined by main roads in the city. The Portland Community Clinic resides in CHA#2. CHA#2 experiences one of the country's highest incidences of chronic alcohol addiction, smoking, drug use, HIV/AIDS, HPV, poor diet and poor oral hygiene, making the population at high-risk for developing OSCC if they live long enough.

The rate of HIV infection in Vancouver is the highest nationwide, with 212 newly reported cases of HIV in 2003 compared to 7 in Richmond (Vancouver population ~578,000. Richmond population ~176,000). Approximately 90% of these newly reported HIV cases in Vancouver occurred within the DTES (McLean, 2002). With respect to OSCC, HIV infection increases the relative risk of oral cancer-related mortality by 2.0 in males and 4.3 in females (Kreimer *et al.*, 2005). This elevated risk stems from a cascading effect of HIV-induced immuno-compromisation leading to oral HPV infection and Karposi sarcoma. Susceptibility to infection depends on the individual's current serostatus when infected with HIV. For reference, Appendix 7.4 shows the incidences of hepatitis B and C, tuberculosis and HIV in the Portland Community Clinic. Similar data was not gathered for the Abacus Dental Clinic.

The DTES region is the poorest region in Canada; based on Canada's 1996 census, approximately 68% of residents were classified as low-income, based on an annual earning of less than $11,029 Canadian dollars, compared with the city average of 31% (McLean, 2002; Statistics Canada, 2001). The actual percentage is estimated to be higher due to the exclusion of many homeless individuals who also live in the area. Low income is associated with a poor diet. During preliminary data collection, patients were assessed on their overall dietary intake, which was later excluded from the final questionnaire assessment. The degree of detail required was difficult for many patients to recall and the additional time to respond made many patients unwilling to complete the questionnaire. During this preliminary diet assessment, the most commonly reported statement was "Sometimes I did not have enough to eat" and "Had enough to eat, but not

always the kinds of food I wanted" with an average fresh fruit and vegetable intake of 2-4 times per week.

Tobacco and alcohol use are also more common in DTES although the average cigarette consumption is not significantly different than in the ADC. This is mainly due to financial constraints that limit cigarette consumption in DTES. As an alternative, DTES residents also turn to cheaper, longer-lasting illicit drugs such as marijuana, cocaine and methamphetamine. It is interesting to note that methamphetamine is a strong appetite suppressor and users may not feel the need to eat for several days, severely compromising an individual's health. Intravenous drug use is also rampant in this region and many individuals do not utilize local safe injection sites (Kerr *et al.*, 2003), resulting in the proliferation of diseases such as hepatitis and HIV and thus, severely impacting the ability of an individual to recover from illness, infections or chemical assault such as metabolism and excretion of smoking-related carcinogens. Drug use itself also adds to the xenobiotic body burden of an individual.

Table 2 shows that the PCC group has an average of 19.7 tobacco pack-years (~144,000 cigarettes) compared to the Abacus dental clinic (ADC) group of 13.9 pack-years (~101,000 cigarettes). In addition, the DTES has a high incidence of crack cocaine smoking. Crack cocaine smoking's relationship to oral cancer lacks scientific evidence; however, many known carcinogens exist in crack cocaine smoke and histopathological abnormalities such as hyperplasia and squamous cell metaplasia have been found in crack cocaine smokers compared to non-cocaine smokers (Fligiel *et al.*, 1997; Mao and Oh, 1998).

During preliminary studies from the screening program conducted at the PCC of 133 patients, we found 26 cases of clinically significant leukoplakia resulting in 6 biopsies. Four were found to show oral premalignant dysplasia with an additional single case of oral cancer (Poh *et al.*, 2007). This is an extremely high rate based on the average of <1% for leukoplakia for North American adults over the age of 40, with approximately 6-31% of leukoplakia cases becoming cancerous, based on Table 1.

Based on the epidemiological data and screening results from this study, we classify the DTES population as high risk for the development of OSCC. However, cancer is an age-related disease. The average life expectancy in Vancouver is 81.1 years, but the assessed life expectancy in the DTES is 60 years (BC Stats, 2005; Gilmore, 2004). Low income, non-drug users in the DTES have a longer life expectancy compared to drug users, particularly juveniles and adults under 30 years of age, who experience a higher rate of accidental deaths such as drug overdose, homicide and suicide (Miller *et al.*, 2007). Although the population is high-risk, many who are at risk do not live long enough to develop OSCC.

1.5.3 Problems in Clinical and Histological Diagnoses of OPLs and Cancers

Although late-stage cancer is easily diagnosed, clinical presentation of premalignant lesions and early cancer are frequently not unique to the disease and can be easily confused with common inflammatory oral lesions. Some of the oral premalignant lesions and early cancers are not even clinically visible. Hence, diagnostic biopsies are frequently not taken early in the disease development, when intervention is more likely to be successful.

Histologically, even when such a lesion is biopsied and submitted for examination, difficulties occur with the current histological standard for predicting the outcome of oral premalignant lesions. The grading of degree of dysplasia requires an experienced pathologist and is still highly subjective. Furthermore, histological grading has a poor predictive value with respect to prediction of risk of malignant transformation for lesions with no or minimal dysplasia. The latter represent the bulk of OPLs that are biopsied (Poate *et al.*, 2004; Warnakulasuriya, 2001).

Finally, the inability to differentiate which early lesions are more likely to progress to cancer makes treatment decisions for OPLs difficult. Currently, there is no universally accepted standard for treatment for OPLs, particularly for those with minimal degrees of dysplasia. Hence, in many cases, a decision is made to "wait and see" rather than to treat; waiting for the lesion to develop to a later stage before intervening. An additional complication arises even when a decision is made to surgically remove such a lesion. It is not uncommon for invasive and pre-invasive cancerous cells to spread beyond the clinically visible borders of a lesion. These cells may grow outwards from a lesion as finger-like projections in single file, masked from clinical appearance by the surrounding normal tissue (Upile *et al.*, 2007). Surgeons usually add 1 cm into clinically normal margins around cancers in an attempt to catch such extensions, but no such concensus exists for OPLs. However, approximately 25% to 48% of oral cancers treated by surgery with or without adjuvant radiotherapy recur, suggesting that residual cells are left behind at the treatment site (Koo *et al.*, 2006). The high mortality rate associated with oral cancer results from a combination of late detection and local recurrence.

In summary, current clinicopathological problems related to diagnosis of OPLs and early OSCC have resulted in the late diagnosis of OSCC, and the dismal prognosis for the disease. Tools and molecular markers that can distinguish high-risk from low-risk OPLs are highly desired. Molecular markers currently have a major emphasis in tumour marker research.

1.6 Molecular Identification of High-Risk OPLs

The identification of molecular markers that target genes or chromosome abnormalities associated with cancer and its development represents a new branch of research aimed at early detection and diagnosis. Research has shown that carcinogenesis is a multi-stage process that, requires the accumulation of multiple genetic mutations alterations (Fearon and Vogelstein, 1990). There is some indication that an order or sequence of genetic mutations may affect cancer development with some gene regions being associated with early mutational loss, while other changes occur most frequently at late stage events (Croce *et al.*, 1999; Kitamura *et al.*, 2001).

Animal models have been used to separate key events involved in carcinogenesis into three stages: initiation, promotion and progression. The initiation of cancer begins within a single cell, where a mutation gives it growth or survival advantages over adjacent cells (Fialkow, 1979; Scholes *et al.*, 1998; Sidransky *et al.*, 1992). This results in the production of clones of genetically homologous daughter cells that also contain the initial mutation. The process of clonal expansion is facilitated by the action of promoting agents that act through epigenetic processes to facilitate the selective outgrowth of initiated cells (Bedi *et al.*, 1996; Fialkow, 1979).

Progression involves the further accumulation of genetic change to cells within the initiated clone. Over time, these cells become heterogeneous, often with elevated rates of genetic instability acquiring genetic alterations that allow them to invade and metastasize to other tissues.

During the last two decades, scientists have developed biological models that integrate key molecular changes with histology to describe the process of carcinogenesis at different tissue sites. The first such molecular progression model was developed by Vogelstein (1988) for colorectal cancer. In this model, a minimum of 4 genetic events was required for colorectal cancer development. This approach has evolved over time to include a variety of other cancers. In oral cancer, it is believed at least 6 to 10 genetic events are required for cancer development (Todd *et al.*, 1997). The different number of genetic events for a cancer is highly dependent on the site. Tissues of the stomach, liver and colon normally have high cell turnover rates, and require fewer genetic events to induce cancer. Tissues such as nerves and muscles do not proliferate and, thus require a greater number of genetic events to inactivate growth suppressing; or activate growth-promoting genes.

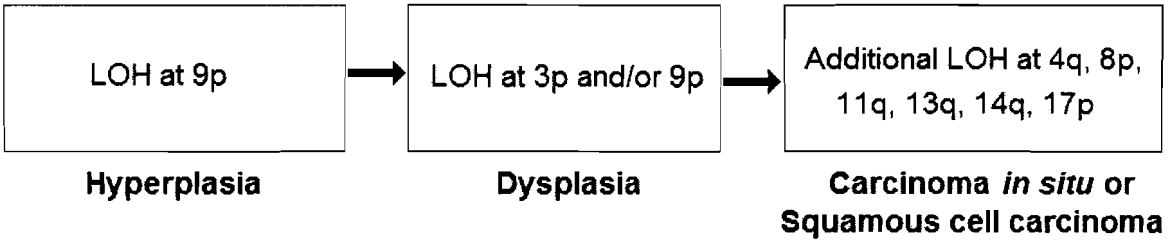
For oral cancer, early genetic mutations often involve the inactivation of tumour suppressor genes (TSG) such as the fragile histidine triad (*FHIT*), *p53* and *p16* and/or overexpression of growth regulating molecules such as cyclin D1 and epidermal growth factor receptor (Gillenwater *et al.*, 2006). The molecular changes within a cell occur prior to and during morphological changes, thus they precede and overlap the events described in the histological progression model.

The oral cancer molecular progression model was first proposed by Califano *et al.* (1996). In the proposed model, they suggested a frequent pattern in the sequence of alterations that occurred in association with the histological progression of this disease. They noted that loss of heterozygosity (LOH) at *9p* was the earliest event, occurring in hyperplasia; that *3p* and 17p were associated with dysplasia; and that 4q, 6p, 8p, 11q, 13q and 14q represented losses more frequently observed in CIS and OSCC. LOH studies detect the loss of one parent's contribution to part of the genome of a cell. Often in cancer, a tumour suppressor gene is absent in the lost section of the genome. A functional second copy of the gene normally exists, but in cancer, this backup copy is often inactivated by a mutation (Ishwad *et al.*, 1996).

1.6.1 Identification of High-Risk OPLs using Molecular Markers

In several landmark studies, research has shown that low-grade dysplastic lesions with specific LOH patterns have a significantly higher relative risk for oral cancer compared to lesions without these patterns (Mao *et al.*, 1996; Rosin *et al.*, 2000). These studies showed that LOH analysis was able to differentiate histologically similar lesions with little likelihood of malignant transformation from those with a greatly elevated risk (relative risk over 30-fold) (Rosin *et al.*, 2000). A key change that separated such lesions involved the presence or absence of LOH at *9p21* and *3p14.2*. Such alterations were the earliest events in the molecular progression of lesions, occurring in almost all lesions that later progressed into cancer. Currently, research in our laboratory is testing the predictive value of these LOH patterns. This research has led to the proposed molecular progression model of OSCC shown in Figure 5.

Figure 5 Proposed molecular progression model of oral squamous cell carcinoma



*Adapted and modified from Mao *et al.* (1996), Rosin *et al.* (2000)

The proposed molecular progression model of OSCC is based on the order of cumulative chromosome loss. Loss of the *9p* chromosome is frequently the earliest event and is associated with hyperplasia. Loss of *3p* with or without *9p* is found frequently in dysplasia. Additional loss of a combination of chromosomes *4q, 8p, 11q, 13q, 14q* and *17p* are more frequent in CIS and OSCC.

LOH assessment and other molecular markers may eventually become a powerful adjunct tool for differentiating low-risk, low-grade dysplasia from high-risk, low-grade dysplasia. In this thesis, a different technique, fluorescence *in-situ* hybridization was used to identify gene and chromosome changes that may serve as an alternate or complementary approach to risk assessment. The following sections will provide background to this approach, including a description of the role of chromosome instability in cancer and then focusing on the regions that will be studied with FISH and the genes that may be important to OSCC development in these regions: *3p14.2* and *9p21*.

1.7 Chromosome Instability as a Biomarker

Cellular manifestations of cancer occur after genetic changes. Genetic changes can be classified into four categories: 1) nucleotide changes (e.g., deletions, base changes, additions), 2) changes in chromosome numbers (e.g., aneuploidy, polysomy), 3) chromosome translocation (e.g., familial translocation), and 4) gene amplification

(Lengauer *et al.*, 1998). These changes result in the loss of TSGs and the alteration of oncogenes that underlie the process of carcinogenesis.

Growing lines of research suggest that genetic instability is a critical step that makes DNA increasingly susceptible to the accumulation of genetic damage. Normal mutation rates in DNA are very low, estimated at $<10^{-8}$ due to various DNA repair mechanisms in the cell (Tomlinson *et al.*, 1996). Given that approximately 6 to 10 critical gene mutations must occur in carcinogenesis, it has been suggested that the normal rate of mutation cannot accomplish this (Todd *et al.*, 1997). Therefore, a mechanism likely exists to accelerate the mutational rate.

Two main forms of genetic instability have been examined widely in humans: microsatellite instability and chromosomal instability. Microsatellite instability is associated with mutations in mismatch repair genes whereas the process of chromosomal instability is complex and currently not well understood (Fodde *et al.*, 2001; Lawes *et al.*, 2003). Changes in chromosome or centromere numbers and unbalanced chromosome translocations are hypothesized to be the two primary mechanisms for chromosome instability (Lengauer *et al.*, 1998). Alterations of chromosome numbers leads to a condition known as aneuploidy, where cells do not contain the normal complement of chromosomes. Recent studies suggest that aneuploid cells cause chromosome instability (Duesberg, 2005). Several studies suggest that alterations to tubulin and other cytoskeletal structures such as the centrosome or centromere occasionally and randomly misdirect chromosomes during mitosis resulting in cells with abnormal quantities of genetic material. The loss or gain of chromosomes leads to aneuploidy and chromosomal instability. Biologically, aneuploidy results in the dysregulation of gene products on the

chromosome that are either lost or increased in copy number (Chial and Winey, 1999; Saunders *et al.*, 2000).

Chromosome translocation, which is the abnormal transfer of genetic material between non-homologous chromosomes, is the second mechanism believed to induce chromosome instability. The translocation process may be unbalanced, resulting in a loss of genetic material during transfer and potentially causing the loss of genes related to the development of chromosome instability such as tubulin and cytoskeletal regulatory genes (Saunders *et al.*, 2000). Unbalanced chromosome translocations are also shown to cause double-stranded DNA breaks. A region of the genome that regularly undergoes unbalanced chromosome translocations is the fragile histidine triad (*FHIT*) described in section 1.7.2. It becomes structurally weaker and more susceptible to carcinogenic chemical assault and hence prone to such translocations events (Arlt *et al.*, 2006; Ferguson and Alt, 2001; McClintock, 1942). Unbalanced translocations delete vital tumour suppressor genes such as those in the *FHIT* region, promoting the development of tumours. Regardless of the mechanism by which instability arises, the hypothesis that aneuploidy or chromosome instability is a precursor to the development of cancer is gaining acceptance in the scientific community (Gollin and Skarja, 2006).

Groundbreaking studies in 2001 and 2004 by Dr. John Sudbo suggested that the degree or severity of aneuploidy may potentially be used as a prognostic marker of oral cancer risk, to predict the aggressiveness of the disease for cancer patients and also to identify individuals with OPLs that are at elevated risk of developing OSCC. This research played a vital role in the development of subsequent research projects regarding ploidy and cancer development around the world, including this project. Although Dr.

Sudbo's data was found to be falsified in 2006, and the work was retracted (Couzin, 2006), the ideas reported were highly plausible and were supported by past and present research results that were unrelated to Sudbo's (Couzin, 2006). Ploidy assays measure total DNA content within cells and thus, the degree of aneuploidy of a sample. The degree of aneuploidy is proportional to the severity of genomic instability, providing a mechanism for accelerated mutational rates (Duesberg *et al.*, 1998). High numbers of aneuploid cells prior to any histological changes may provide insight on an individual's risk of accumulating further mutations leading to cancer. It is important to note that although promising, future research is required to support the association, to indicate how generalizable it is to different populations and to better develop the protocol used to determine aneuploidy. For example, there is no established cut-off value for the percentage of aneuploid cells in a sample that would constitute a significant elevation in risk.

Unlike detection of chromosome instability, the loss of specific tumour suppressor genes has been established as an effective method to quantify the risk of developing oral cancer. Within some chromosomes lie regions that are inherently more susceptible to DNA damage. For example, gene regions such as *3p14.2* lie within fragile sites that are more frequently mutated, inactivated or completely lost in various stages of OSCC (Huebner and Croce, 2003; Rosin *et al.*, 2000). Fluorescence *in-situ* hybridization provides a useful tool to quantify alterations to chromosome numbers and gene copies in the same cells.

1.7.1 The *9p21* Locus

Based on the molecular progression model of OSCC, the number of genetic mutations in critical genes provides the strongest association with an individual's risk of developing OSCC. The sequence of molecular change is also important and *9p21*, a 30.1MB region, has been identified as a genetic site that is often deleted or inactivated in the early stages of oral carcinogenesis. Identical frequencies of LOH at *9p* in invasive and pre-invasive head and neck cancer suggests these changes occur prior to CIS (van der Riet *et al.*, 1994). Frequent microsatellite deletion at *9p* in precancerous lesions also support *9p* loss in premalignant lesions (Mao *et al.*, 1996). *9p* loss is also found to be present in early lesions at other sites and associated with risk of progression, for example, esophageal lesions that progress to cancer frequently show loss on *9p* (Wong *et al.*, 2001).

The *9p* region is commonly deleted in hyperplasia and low risk dysplasia cases indicating the loss occurs early in the sequence of mutations leading to cancer (Mao *et al.*, 1996; Rosin *et al.*, 2000). Loss of *9p21* has also been associated with recurrence of OSCC after treatment or surgical elimination of the primary cancer, and with lower survival rates (Lydiatt *et al.*, 1998; Rosin *et al.*, 2002). Thus, the detection of *9p21* loss may provide insight into a person's risk of developing OSCC, as well as recurrence of the disease.

Epigenetic changes in the *9p21* region have also been implicated in cancer development. Hypermethylation of a promoter region inhibits gene transcription and for *9p21*, promoter hypermethylation results in gene inactivation, phenotypically equivalent

to gene loss. Hypermethylation of *9p21* has also been implicated as a common early event in OSCC (Shintani *et al.*, 2001; Weber *et al.*, 2003).

The *9p21* locus encodes two TSGs responsible for negative regulation during the cell cycle. These genes, $p16^{INK4a}$ and $p14^{ARF}$ are involved in regulating growth control pathways in the retinoblastoma (RB) and *p53* apoptosis pathways, respectively. Functional inactivation of retinoblastoma and *p53* pathways is considered an essential step in almost all human cancers as these events disrupt cell cycle regulation and deactivate the apoptotic response (Chin *et al.*, 1998).

RB protein plays an integral in the cell cycle by binding and inhibiting the E2F-transcription factor (William and Kaelin, 1999). This constitutes a critical inhibitory pathway of cell proliferation at the restriction point (R-point) of the cell cycle, between the G1-growth phase and the S-phase. The R-point represents the cell's irreversible commitment to replicate, beginning with the synthesis of DNA for the daughter cells during mitosis. Under normal conditions that promote cell replication, the complex of cyclin-dependent kinase 4 (CDK4) and cyclin D act to phosphorylate RB which detaches it from the E2F-transcription factor allowing transcription of genes promoting cellular transition to S-phase (William and Kaelin, 1999). The protein product $p16^{INK4a}$ is a negative regulator the CDK4-cyclin D kinase activity. Thus, inactivation of $p16^{INK4a}$ allows for unregulated kinase which, promotes RB detachment from E2F-transcription factor, allowing E2F to induce transcription of cell proliferative gene products (Chin *et al.*, 1998).

p53 is a TSG that is believed to be the most commonly mutated gene in carcinogenesis and considered a required event in almost every cancer. The expression of

p53 protein normally induces $p21^{CIP1}$-mediated cell-cycle arrest and, under specific conditions, programmed cell death (El-Deiry, 1998; Vogelstein and Kinzler, 1992). In the presence of the protein MDM2, *p53* binds to it and begins to degrade, lowering cellular concentrations. $p14^{ARF}$ protein binds to MDM2, causing the release of *p53* protein, thereby stabilizing *p53* levels allowing cell-cycle arrest. The superscript of $p14^{ARF}$ refers to the term "alternative reading frame" of the *9p21* locus. Both $p16^{INK4a}$ and $p14^{ARF}$ mRNA are transcribed from the same gene region. However, through a series of complex exon splicing events, $p16^{INK4a}$ mRNA is transcribed from one DNA strand in the 3' to 5' direction while $p14^{ARF}$ mRNA shares the first exon with $p16^{INK4a}$ but the remaining two exons are from the alternative, anti-parallel DNA strand spanning the 5' to 3' direction (Kamijo *et al.*, 1997). This allows the *9p21* locus to encode two distinctly different gene products, which utilize completely different pathways. It also suggests that any mutation to this locus also has the potential to inactivate both TSGs simultaneously.

Alterations in *9p21* as a result of tobacco use are well documented. Tobacco-related carcinogens cause microsatellite instability and LOH in OSCC patients at the *9p* locus in tobacco-consuming East Indian populations (Mahale and Saranath, 2000). In a study of precancerous tumours that progressed to OSCC, homozygous deletion of $p16^{INK4a}$ and $p14^{ARF}$ were found in 26.5% and 56.3%, respectively. Furthermore, 43.8% of $p16^{INK4a}$ and 50% of $p14^{ARF}$ exhibited aberrant methylation of their promoter regions. Cumulatively, 87.5% of all OPL samples contained some form of inactivation of the *9p21* locus, supporting the theory that this is a suitable molecular marker of higher OSCC risk (Shintani *et al.*, 2001). However, polysomy, the presence of more than 2 copies of a specific chromosome (a type of aneuploidy) is also believed to be a risk factor. In an

analysis of *9p* polysomy, the polysomy index of biopsy samples increased significantly from 1.4 in low-risk leukoplakia lesions with mild dysplasia to 2.1 in high-risk leukoplakia with moderate dysplasia (Kim *et al.*, 2001). Dysplastic tissue showed even greater polysomy levels relative to hyperplasia. Interestingly, biopsied tissue with both hyperplastic and dysplastic regions showed a two-fold increase in polysomy in the hyperplastic region from 1.3 to 2.6, suggesting that polysomy itself may also be an early indicator along with *9p21* locus inactivation (Kim *et al.*, 2001).

The *9p21* region is also known to contain genes encoding other proteins that are related to tumour development and cancers at other sites. One such gene is "a disintegrin-like and metalloproteinase with thrombospondin" (ADAMTS) protein (Hall *et al.*, 2003; Porter *et al.*, 2005).

1.7.2 The *3p14.2* Locus

The *3p14.2* chromosome locus contains a structurally fragile region in which the Fragile Histidine Triad (*FHIT*) gene resides. This gene is approximately 1Mb in size with the entire *3p14*.1-*3p14*.3 region being 55.6 Mb (Zimonjic *et al.*, 1997). The *3p14.2* locus was first identified as containing a translocation breaking point in familial renal cell carcinoma over 28 years ago (Cohen *et al.*, 1979). The involvement of *3p14.2* deletion from t (3:8) translocations has since been implicated in cancer-derived cell lines of the lung, stomach, pancreas, esophagus, cervix and colon (Hadaczek *et al.*, 1999). Today, the evidence is irrefutable that *3p14.2* change is a frequent and potentially important alteration in a multitude of cancers. Ironically, in spite of the abundance of research showing hypermethylated inactivation of promoter sites (Puri *et al.*, 2005), loss of heterozygosity and polysomy for this region (Ishwad *et al.*, 1996; Pekarsky *et al.*, 2002;

Rosin *et al.*, 2000; Rosin *et al.*, 2002), the protein functions of genes within it are still largely unknown. Structurally, the *FHIT* gene codes for a protein that resembles the yeast enzyme diadenosine tetraphosphate hydrolase, which belongs to a family of enzymes responsible for binding nucleotides known as histidine-triad nucleotide binding proteins (Huebner *et al.*, 1998). A range of ADAMTS proteins are also encoded in this region and are known to be lost in hereditary renal tumours (Clark *et al.*, 2000).

Fragile sites have been implicated in numerous cancers due to increased susceptibility of these regions to chemically induced chromosome breaks. Genes within *FHIT* are often mutated, deleted or under-expressed in various cancers including oral and other head and neck sites. Experiments that knockout or underexpress *3p14.2* ultimately induces tumour formation. The replacement of various *3p14.2* segments through gene therapy restores normal cell function, suggesting more than one tumour suppressor gene is localized in *3p14.2* (Dumon *et al.*, 2001).

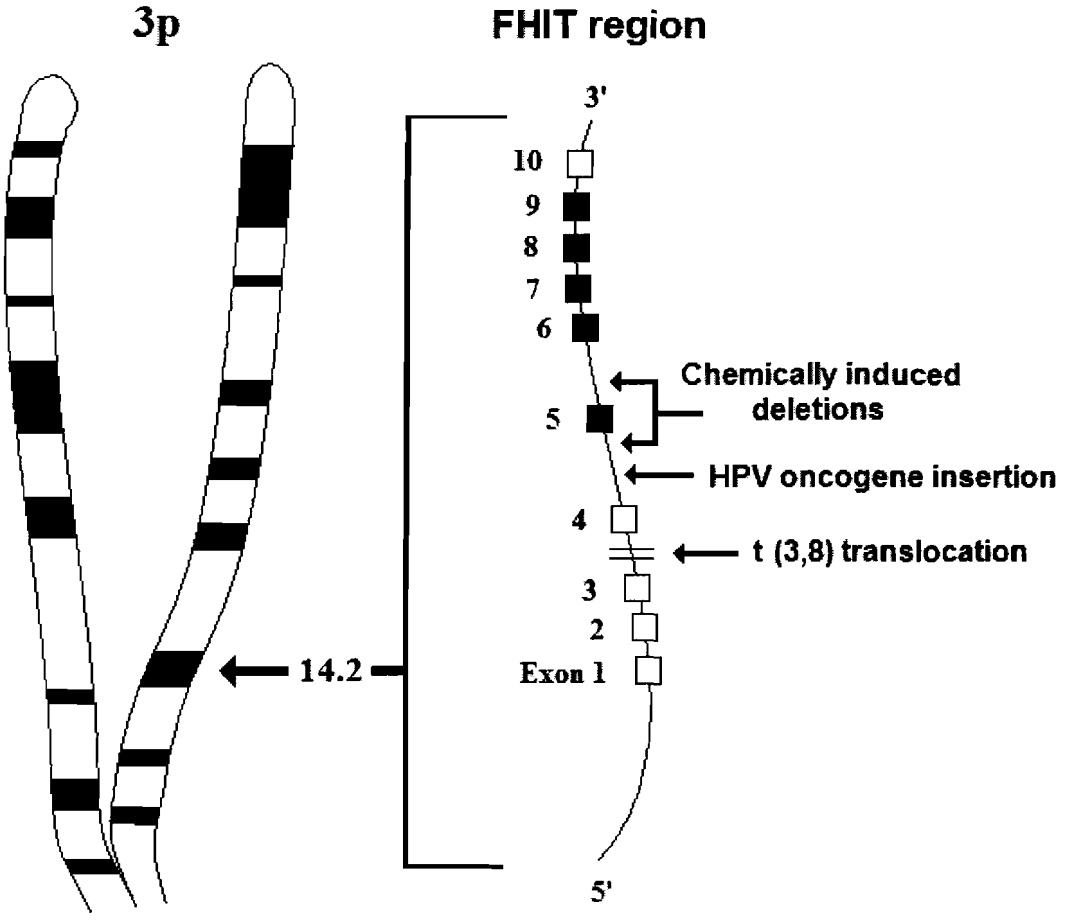
There are approximately 103 fragile sites identified in all human chromosomes (Zimonjic *et al.*, 1997). Some are common, rare or inherited. However, they share a common feature of having G-C rich regions encoding Alu restriction endonuclease repeats and are generally under-coiled and over-relaxed during DNA transcription. At these regions, the DNA is more susceptible to strand breakage, translocations and recombinations relative to its stable relaxed state (Manuelidis, 1990). *FHIT* is a common site of familial translocation in which non-homologous chromosomes exchange genetic material. Unbalanced translocations fragment the region, inactivating the suspected tumour suppressor functions of the genes. Balanced translocations, where there is no loss

of genetic material, can also be detrimental as gene become underexpressed even with intact gene function due to translocation beside a weak promoter region.

Additionally, some human papilloma virus (HPV) strains insert viral DNA into the host genome within the region of *3p14.2*, affecting the function of the normal gene products (Huebner *et al.*, 1998). Figure 6 shows a representation of the *3p14.2* chromosome band with the *FHIT* locus and the regions within it that are susceptible to mutations including chemical mutagen-induced deletions, familial translocations and HPV-induced integration of oncogenic DNA.

Figure 6 Illustration of the Fragile Histidine Triad region

Illustration of the *FHIT* region of the *3p* chromosome[1] and the areas susceptible to genetic mutation by chemical induced deletions, HPV oncogene insertion and familial translocation. Black boxes represent transcribed exon regions of the gene and white boxes indicate untranscribed exons.



[1] Adapted and modified from Huebner et. al. (1998)

1.8 Fluorescence *in-situ* Hybridization

Fluorescence *in-situ* hybridization is a technique that hybridizes fluorescently labelled DNA probes with the genome of a target cell. These probes can target virtually any gene or centromere region to quantify the number of gene copies and chromosomes within the cell under a fluorescent microscope. FISH is also used to map the relative position of genes within chromosomes. This process, known as chromosome painting, simultaneously applies multiple FISH probes, each with its respective fluorophore colour to the target genome (Speicher *et al.*, 1996). In the present study, FISH was used to detect the number of gene and chromosome copies in a cell at *9p21* and *3p14.2*.

As mentioned previously, the TSG-containing regions, *3p14.2* and *9p21*, are frequently altered in OPLs and also in non-cancerous dysplasias, suggesting that they are usually mutated early in the sequence of carcinogenesis (Nakahara *et al.*, 2001; Shintani *et al.*, 2001; Uzawa *et al.*, 2001). Loss or inactivation of these regions is also associated with poor outcome and are strong indicators of individuals at risk of cancer recurrence after treatment (Rosin *et al.*, 2002). FISH has also been used to identify genetic loss patterns associated with aggressiveness of potentially malignant oral lesions where 58% of patients had loss at *3p14.2* and/or *9p21* (Poh *et al.*, 2006). FISH has not been validated as an effective risk prediction method in visually normal oral mucosa tissue which, in this study, assesses high-risk regions of the mouth rather than abnormal lesions. Previous studies in our lab have examined FISH signals in cancer patients within tumour margins but the sensitivity of FISH to distinguish between high-risk and low-risk populations with no cellular abnormalities is unknown. Similar studies using LOH have detected allelic loss in low-grade oral dysplasia to help predict the progression or non-progression to

cancer (Rosin *et al.*, 2000), suggesting loss patterns are detectable and identifiable at early stages of development.

Cancer is a genetic disease requiring multiple mutational events prior to morphological changes in the tissue. These genetic changes include TSG gene deletions and genomic instabilities characterized by chromosome loss (Yamasaki and Mironov, 2000); both of which can be detected in cells using FISH. It is postulated that FISH can identify patterns of genetic mutations involved in cancer prior to morphological changes. There is no single gene that controls cancer development, although some genes play more important roles than others. *p53* is an example of a TSG that is mutated or down-regulated in virtually all cancers, demonstrating its importance to normal cell function. However, the loss of *p53* alone does not induce cancer, although it does increase the risk. Patterns of TSG loss that are identified using FISH may help predict individual risk to the development of OSCC.

1.9 Exfoliated Cell Cytology: A Non-invasive Procedure for Collecting Cells for FISH Analysis

One of the impeding factors in detecting OSCC is that current diagnosis techniques require an oral biopsy in which a thin puncture of tissue is obtained from the lesion site. A lesion must be readily visible in order to pinpoint the location for the biopsy. In addition, this invasive technique is uncomfortable, making patients reluctant to have a biopsy without sufficient cause. The fear of pain and discomfort associated with dental examinations already causes many people to avoid or delay dental visits. Recently, the oral brush biopsy has been proposed as a new technique to identify OPLs and OSCC in a non-invasive manner (Kujan *et al.*, 2006; Mehrotra *et al.*, 2006; Rick and Slater,

2003). The technique requires the use of a histobrush, a soft-bristled brush used to scrape high-risk regions of the oral cavity such as the ventral-lateral tongue or the floor of the mouth to collect exfoliated cells. Cells are then spread on a specially prepared slide to be processed and examined by a pathologist. One disadvantage to this technique compared to the standard biopsy is the collection of exfoliated brush cells does not provide information on the tissue structure; exfoliated cells are examined based on cytology rather than histology. The criteria for cytological analysis is similar to histological grading for dysplasia with the exclusion of grading aspects related to whole tissue structure. These include:

1. Increased nuclear and/or cytoplasmic ratio
2. Altered nuclear shape
3. Nuclear hyperchromatism
4. Nuclear and cellular pleomorphism

A review of the efficacy of this technique shows that brush biopsies were capable of distinguishing patients with cellular abnormalities with a sensitivity of 71.4%; however, the specificity to determine the type of abnormality was only 32% (Poate *et al.*, 2004). Similar reviews assess the sensitivity and specificity as 90% and 5% respectively (Rick and Slater, 2003). Therefore, this technique cannot identify all potentially malignant diseases. The analysis is also subjective, requiring a trained pathologist, and interpretations of cell morphology may vary with each pathologist's experience.

Oral brush biopsies using molecular markers identify genetic rather than cellular abnormalities and are less subjective to the variations in pathologist assessments of

cytological and histological morphology. Recent studies have examined the possibility of brush biopsies coupled with fluorescence *in-situ* hybridization (Upile *et al.*) as a risk indicator or diagnostic tool of cancer in a variety of head and neck cancers. Veltman *et al.* (1997) suggested that the presence of chromosomal abnormalities in 5% or more of the sampled cells was an indicator of malignancy. Similar studies have also used bronchus brush biopsied cells in ploidy analysis for risk quantification for lung cancer which shows its usefulness in detecting possible malignancy prior to cellular changes in morphology (Schenk *et al.*, 1997).

It is important to note that other brushing procedures have been used to collect samples for analysis that are even less invasive than the brush biopsy procedure described above. The brush biopsy penetrates deeply into the mucosa and stimulates "pinprick" bleeding, in an attempt to collect the full width of epithelium for assessment. This may not be suitable or necessary for collecting cells from individuals without any clinical lesion. For example, PAP smears have been used to detect cervical cancers with great success and these smears use surface cytobrushes. Cytological analysis of PAP smears, which is similar to oral brush biopsies but more invasive, has reduced the rate of invasive cervical cancers in the United States by more than 70% since its inception into clinical practice over 50 years ago (Walsh, 1998). Clearly, cytology has proven effective as a diagnostic tool for some cancers.

In this study, we employed a simple cytobrush to collect exfoliated cells for analysis. This approach satisfied a key requirement in this study, the ability to implement the procedure within the time constraints of a normal dental appointment and in as non-invasive a fashion as possible. There are multiple variations to obtaining exfoliated oral

mucosa cells including scraping the surface mucosa, rinsing the oral cavity and taking saliva samples. The ideal instrument for obtaining cells should be usable at any location in the mouth, minimize trauma and provide adequate numbers of cells to represent the area of interest. A cytobrush satisfies all of these requirements. It is also inexpensive, risk-free, requires less than a minute to use, and can be conducted while a patient is waiting for a scheduled appointment without delay. Overall, this approach is a faster and non-invasive alternative to tissue biopsies. It also does not carry the hazards of blood-borne diseases associated with handling biopsy samples.

The oral cytobrush procedure is also ideal for visually normal mucosa. Using this procedure on leukoplakia will not yield cells in that region. The surface of a leukoplakia lesion is white due to keratinisation of the cells which, will not exfoliate during the cytobrush procedure.

2. STATEMENT OF THE PROBLEM

Oral premalignant lesions (OPL) are associated with higher risk of developing oral squamous cell carcinoma (OSCC), but only a portion of these lesions will progress into cancer. Currently, clinicopathological risk factors are used to identify high-risk OPLs and early OSCC. However, many problems exist with current clinicopathological risk predictions, thus, new tools are needed to help in the identification of high-risk lesions.

Like other cancers, OSCC is a disease that must be preceded by the accumulation of genetic mutations. Molecular markers that identify genes associated with cancer progression may accurately quantify OSCC risk in healthy patients and improve early detection rates prior to OPL development, and also predict outcome of existing OPLs. The loss or inactivation of tumour suppressor genes (TSG) at *3p14.2* and *9p21* has been identified as an early mutational event in the progression of OSCC. Thus, they are both candidate molecular markers to predict individuals at high risk for developing OSCC. Currently, no such marker has been applied in clinical practice to screen at-risk individuals. Such a test would ideally be fast and easy to conduct, allowing the procedure to be integrated into regular dental appointments. It would provide clinicians with major advantages compared to the current wait-and-see approach by providing information to assess an individual's risk of developing OSCC prior to the development of OPLs.

3. HYPOTHESIS AND OBJECTIVES

Genetic alterations in the tumour suppressor genes at *3p14.2* and *9p21* are early indicators of OSCC risk. Tobacco use, alcohol consumption, illicit drug use, diseases and poor diet are all significant risk factors in the development of OSCC. The Downtown East-side population in Vancouver, British Columbia, Canada, significantly exceeds the national average for tobacco and alcohol consumption, illicit drug use and incidence of diseases related to cancer risk. It is also the poorest region in Canada, with limited access to a healthy diet and lifestyle. This population is therefore considered at high risk for developing OSCC relative to the national average, which is also represented in the high incidence of OPL detected in this population during preliminary data gathering. We hypothesize that this population, represented by PCC patients, will contain a greater proportion of individuals with higher frequencies of detectable genetic abnormalities at *3p14.2* and *9p21* and their respective chromosomes. Compared with a relatively lower risk population from Mission, British Columbia, Canada, specific genetic patterns may be identified which are associated with elevated OSCC risk.

This research project examined the feasibility of FISH to detect genetic patterns associated with elevated OSCC risk. Conducting data and sample collections in a clinical setting also allowed the examination of multiple issues associated with clinical practice. The objectives of this study are described below:

1. To identify specific patterns of genetic change within *3p/3p14.2* and *9p/9p21* that are present in individuals with an elevated risk of OSCC development based on

epidemiological criteria and comparing them to a population presumed to have a lower proportion of high-risk patients.

2. To provide patients at elevated risk of OSCC with free oral cancer screening.
3. To determine the acceptability of the oral cytobrush collection procedure among dentists and patients by using this non-invasive cell sample collection method.
4. To determine the ease of integrating the oral cytobrush technique procedure into a regular dental visit.

4. MATERIALS AND METHODS

4.1 Patients

4.1.1 Ethics Approval and Questionnaire

Ethical approval for this research was obtained from the ethics committees at Simon Fraser University, The University of British Columbia and the British Columbia Cancer Agency (Appendix 7.1). Patient participation was voluntary; however, only individuals who completed the questionnaire were included in the study.

To ensure accurate interpretation of the questionnaire by the patient, questions were read aloud and explained to the patient and the interviewer recorded the answers.

4.1.2 Study Groups

There were two comparison groups:

1) High-risk group: 53 dental patients from Portland Community Clinic (PCC) in Vancouver, B.C.'s Downtown East-side, which has a higher presumed risk of developing OSCC based on preliminary screening data from a study of 133 patients, with 26 case of leukoplakia, 4 of which were oral premalignant dysplasia and 1 case of OSCC (Poh *et al.*, 2007).

2) Average-risk patients: 51 dental patients from Abacus Dental Centre (ADC) in Mission, B.C.. These patients have a presumed average risk of developing OSCC relative to the PCC population based on previously gathered data related to this study with no incidence of OSCC and no cases of high-risk dysplasia.

4.1.3 Selection Criteria

Patients were required to meet the selection criteria to be included in this study. The selection criteria for the PCC group was:

1. Patients must provide a signed consent to be included in the study.
2. Patients must be at least 35 years old.
3. Patients must be residents of the Downtown East-side, defined by the postal code prefix "V6A" or "V6B".
4. Patients do not currently have oral cancer or any visible abnormalities in the oral mucosa resembling the oral premalignant lesion leukoplakia or erythroplakia.

In the ADC group, the selection criteria was:

1. Patients must provide a signed consent to be included in the study.
2. Patients must be at least 30 years old.
3. Patients do not currently have oral cancer or any visible abnormalities in the oral mucosa resembling the oral premalignant lesion leukoplakia or erythroplakia.

4.1.4 Portland Community Clinic (PCC)

PCC patients were defined as high-risk based on multiple factors. Many of these factors cannot be quantitatively assessed for their influence on OSCC development, and the complexity of interactions allows for only a generalized definition of "high-risk".

Based on preliminary data described in section 4.1.2, the PCC group contains an elevated proportions of high-risk patients compared to the Abacus Dental Centre.

4.1.5 Abacus Dental Centre (ADC)

Patient data and samples were collected at the Abacus Dental Centre (ADC), in Mission, British Columbia. The ADC group is classified as average-risk patients representative of the general population in Canada. This population falls primarily within the middle income and socio-economic range and is not expected to carry the same extremes in dietary, disease and drug use burden that is associated with the DTES population. Patients must also not have any clinical signs of OSCC.

4.2 Data and Sample Collection

Each patient was assigned a path number, which was used to track his or her identity during the study, and to allow study analysis without knowledge of patients to avoid bias.

4.2.1 Demographic Data

All patients were required to complete a detailed questionnaire (Appendix 7.3). and the following information was recorded for PCC patients:

1) Demographic information: age, gender, ethnicity

2) Habit information: smoking habits, alcohol consumption, illicit drug and prescription drug use

3) Health information: history of diseases, primarily diabetes, hepatitis, HIV and tuberculosis

4) Other information: annual income, dietary habits, familial incidences of head and neck cancer

The ADC group was assessed prior to the development of this study. The original study intended for this population did not examine factors such as total alcohol consumption, illicit and prescription drug use, incidence of disease and dietary habits.

Tobacco consumption was quantified in pack-years, which is a measure of total tobacco exposure regardless of age. Refer to Appendix 7.2 for a sample calculation of pack-years. Alcohol consumption, defined as consuming in excess of 3 alcoholic beverages annually, was collected for both groups, although a more detailed investigation of the estimated total alcohol consumption was conducted for the PCC group only.

4.2.2 Visualization Tools

As part of the screening program, two visualization tools were used prior to sample collection that were not directly examined in this study. First, a specialized device called a Visually Enhanced Lesion Scope (VELScope) emitting a blue light was directed into the oral cavity of each patient. The VELScope is referred as a Goggle light in the oral health questionnaire in Appendix 7.3. This light is capable of penetrating the surface epithelium of the oral cavity to the basal lamina. Upon exposure of the basal lamina to the blue light, it emits a fluorescent light that is visible with specialized filter glasses (Kois and Truelove, 2006). Regions that have a thicker epithelium than normal as a result of infection, inflammation or stages leading to cancer such as hyperplasia or dysplasia will not allow the light to penetrate to the basal lamina resulting in a region that does not fluoresce and appears black. This tool is currently being developed as part of this study

and is not clinically proven to be effective at detecting OSCC. Secondly, a toluidine blue staining test was given to the patients. Toluidine blue, when exposed to the oral mucosa, penetrates cell membranes and binds to nuclear material. Regions with darker staining indicate regions with increased DNA content, a predictor of possible cancer development (Silverman *et al.*, 1984b).

4.2.3 Exfoliated Cell Collection

Patients rinsed their mouths with tap water prior to sample collection to remove food and other debris from the oral cavity.

For PCC patients, a cytobrush was used to brush the ventral surface of the entire tongue. Brushing was done in strokes parallel to the length of the tongue and moved from the left ventral-lateral to the right ventral-lateral side. The cytobrush was immediately placed in a vial containing 1ml of PreservCyt supplied by Cytyc Corp. (Marlborough, MA, USA); a methanol based buffer solution formulated to eradicate infectious agents. The cytobrush was spun vigorously to transfer exfoliated cells into the vial and stored at 4°C. Two brush samples were collected for the ventral-lateral tongue per patient.

ADC patients were asked to brush their teeth prior to sample collection if they did not have their teeth cleaned during their dental appointment. The same cell collection procedures were used except cells were immersed in 1ml TE-9 buffer solution (0.03M Tris, 3.0mM EDTA at pH 8.9) instead of PreservCyt and snap frozen in liquid nitrogen. Cell samples were transferred to PreservCyt and stored at 4°C within 6 hours. One sample was collected from the ventral-lateral tongue per patient.

4.3 Laboratory Procedures

4.3.1 DNA Probes

This study used four fluorescently labelled DNA probes, which were either pre-made or created using the Nick Translation Kit, both obtained from Vysis (Downers Grove, IL, USA). Chromosome enumeration probes (CEP) 3 and 9 were fluorescently labelled with Spectrum Green fluorophores to target centromeric regions of chromosome 3 and 9, respectively. Spectrum Orange was used to target the *3p14.2* gene region on chromosome 3 and the *9p21* gene region on chromosome 9. Centromeres are the central region of a chromosome responsible for joining the two chromatids of a chromosome together and binding to spindle fibres during mitosis and meiosis. Centromere loss or amplification is indicative of an equivalent change in the number of chromosomes because chromosomes cannot exist without centromeres, which play vital roles in directing chromosomes and cell cycle checkpoints (Choo, 1997). Prior to nick translation, BAC plasmids were purified using a Nucleobond BAC purification kit supplied by Macherey-Nagel (Düren, Germany) and stored at -20°C.

4.3.2 Nick Translation - CEP3/*3p14.2* Probe

Nick translation produces fluorescently labelled DNA probes, which bind to specific nucleotide sequences corresponding to a target gene that were designed in this study to be approximately 600 base pairs. Each labelled probe fragment is unique, and multiple probes will bind to the target gene within a single cell. Nick translation was used to produce the CEP3 and *3p14.2* fluorescent probes only. Nick translation reagents and the completed probes are light sensitive; therefore, entire process of nick translation was

conducted in a low-light environment. The procedure utilizes two strains of *Escherichia coli* bacteria containing the bacterial artificial chromosome (BAC) with gene inserts for CEP 3 and *3p14.2*. BAC strain 170K19 contains the gene sequence for *3p14.2* while BAC 91A15 contains the CEP 3 sequence. The BAC DNA acts as a template for DNA synthesis during nick translation, which incorporates fluorescently labelled uracil triphosphate (UTP) into the new DNA strand. The purified and fluorescently labelled DNA can then be heat denatured causing strand separation. It is then applied to the target cells and allowed to hybridize with the target DNA.

4.3.3 Nick Translation Reaction

Three reagent mixtures were prepared according to the manufacturer's specifications. The first solution, labelled "TTP" contained 2µl 0.3mM dTTP with 4µl nuclease-free water. The second solution, labelled "NTP" contained 4µl each of dATP, dCTP and dGTP. The third solution, labelled "UTP" contained 1µl 1mM fluorescently labelled dUTP with 4µl of nuclease-free water. Two mixtures of UTP were prepared; Spectrum Green dUTP was used for CEP3 and Spectrum Orange dUTP was used for *3p14.2*.

The nick translation reaction was conducted in a 1.5ml centrifuge tube. Separate tubes were used for BAC 170K19 and BAC 91A15 containing the *3p14.2* and CEP3 probes, respectively. For BAC 170K19 and BAC 91A15, 4µg and 2µg of BAC DNA was added respectively to each tube and diluted with nuclease-free water for a total volume of 17.5µl each. To each tube, 2.5µl of UTP, 5µl of TTP and 10µl of NTP was added. Spectrum Green UTP was used for BAC 170K19 and Spectrum Orange UTP was used

for BAC 91A15. Next, 5µl of 10X nick translation buffer and 10µl of the nick translation enzyme mixture, composed of DNA polymerase I, II and III was added for a total volume of 50µl per tube. Tubes were incubated in the dark at 15°C for 90 minutes and 150 minutes for BAC 170K19 and BAC 91A15 respectively. After incubation, the reaction was terminated by heat denaturation of the enzyme at 70°C in a waterbath for 10 minutes. The unprecipitated probe was stored at -20°C in a light shielded container until precipitation.

4.3.4 Fluorescent Probe Size Quantification

Nick translation probes bind to target DNA optimally at a size of 500-600 base pairs. To determine the optimal incubation time and probe size for each BAC strain, 9µl aliquots of nick translation reaction were removed every 30 minutes. The reaction was stopped in a 70°C waterbath for 10 minutes. 9µl aliquots were mixed with 1µl loading buffer and run against a 100 base pair DNA ladder (1µl ladder, 9µl loading buffer) and run in an 2% agarose gel (75ml 1X TAE buffer, 1.5g agarose, 12.5µl of 10µg/ml ethidium bromide) at 120V/cm for 45 minutes and visualized under ultraviolet light. The optimal incubation time for producing BAC 170K19 and BAC 91A15 probes was determined to be 90 and 150 minutes, respectively.

4.3.5 Nick Translation Probe Precipitation

Nick translation probes require precipitation and suspension in an appropriate buffer before use. All buffers were supplied in the nick translation kit from Vysis. Although both probes are applied simultaneously to a slide with exfoliated cells, they are precipitated separately. To precipitate the entire 50µl nick translation reaction product,

the following reagents were added in order to a 1.5ml centrifuge tube: 10µl Cot-1 DNA, 6µl salmon sperm DNA, 40µl nuclease-free water, 12µl 3M sodium acetate buffer (pH 5.4) and 300µl 100% ethanol. The mixture was vortexed and cooled to -20°C for 30 minutes and centrifuged at 12500 rpm (16000g) for 30 minutes. The supernatent was discarded and the DNA pellet was rinsed twice with 70% ice-cold ethanol diluted from nuclease-free water and resuspended in 100% ethanol and centrifuged again at 12500 rpm for 30 minutes. The supernatent was discarded and the DNA pellet was allowed to air dry. The pellet was resuspended in 106µl of CEP buffer for CEP3 probe and 106µl of LSI buffer for *3p14.2* probe. The probes were then stored at -20°C in the dark until their use in the denaturing process during FISH.

4.3.6 FISH CEP9/*9p21* Dual Probe

Dual probes for CEP9 Spectrum Green and *9p21* Spectrum Orange were supplied by Vysis. Probes were obtained in a prepared state equivalent to the precipitated nick translation probe and were suspended in CEP or LSI buffer at the recommended concentration.

4.3.7 Fluorescence *in-situ* Hybridization - Slide Preparation

Cell samples were removed from storage at 4°C and tubes were vortexed to re-suspend the cells. 75µl was pipetted out and mixed with 1ml of Carnoy's fixative solution (100% methanol: glacial acetic acid, 3:1 (v/v)). Samples were centrifuged at 7000 rpm (5200g) for 5 minutes and the supernatent discarded. The cell pellet was re-suspended in 300µl of Carnoy's solution and loaded into a Cytospin 3 centrifuge supplied by Thermo Fisher Scientific (Ottawa, Canada), which uses centrifugal force to press exfoliated cells

against a glass slide to maximize cell adhesion and minimize cell loss during processing. Samples were spun at 400 rpm for 10 minutes and allowed to air dry for 1 hour.

4.3.8 Fluorescence *in-situ* Hybridization - Slide Pre-treatment and Aging

Pre-treatment of cell samples on slides is required to facilitate the penetration of FISH probes through the cellular and nuclear membranes to minimize non-specific binding and to denature target DNA to allow hybridization. Slides were aged in 2X sodium citrate buffer at 37°C for 20 minutes to improve signal intensity and hybridization efficiency. Next, slides were immersed in a pepsin solution (49.5ml distilled water, 0.5ml 1M HCL, 25µl 10% pepsin in distilled water (v/v)) at 37°C for 4 minutes to digest cellular and nuclear membrane proteins to allow probes to penetrate the cell. Exact timing for pepsin digestion is critical. Under-digested cells will show little hybridization and over-digested cells will have broken down nuclei resulting in DNA loss. Slides were rinsed twice in 1X phosphate buffered saline (PBS) for 2 minutes each. A fixative solution (41.1ml distilled water, 5ml 10X PBS, 1.35ml 37% formaldehyde, 2.5ml 1M $MgCl_2$) fixed cells on the slide for 3 minutes to preserve the cellular structures and prevent any further degradation during the denaturing process. Slides were rinsed twice in 1X PBS for 2 minutes and dehydrated by immersing the slides in increasing ethanol concentrations (70%, 85%, 100% ethanol) for 1 minute each and allowed to air dry.

4.3.9 Fluorescence *in-situ* Hybridization - Slide Denaturation

Heat denaturation of cellular and probe DNA is required to separate the two strands of DNA. Nucleotides on each strand are bound by hydrogen bonds, which break at temperatures between 72°C and 75°C. To facilitate denaturation, slides were immersed

in a denaturing solution (35ml 70% formamide, 5ml 20X SSC buffer, 10ml distilled water) at 74°C for 5 minutes. After removal from the heated denaturing solution, the DNA can re-hybridize in the presence of water, which aids the reformation of hydrogen bonds. Therefore, immediately after denaturation, slides were quickly dehydrated by dipping them into increasing ethanol solutions (70%, 95%, 100%) and allowed to air dry in the dark.

4.3.10 Fluorescence *in-situ* Hybridization - Probe Denaturation

In a low light environment, FISH probes were heated in a 74°C waterbath for 5 minutes. For nick translation probes, 4.1µl of CEP3 Spectrum Green and 4.1µl of *3p14.2* Spectrum Orange was used per slide. Each probe was denatured in separate tubes and mixed together just prior to application to the slide. For Vysis supplied dual probes for CEP9/*9p21*, 0.8µl of dual probe was mixed with 1.7µl nuclease free water, 2.8µl of LSI and CEP buffer for a total volume of 8.1µl. After denaturation, 8µl of probe was pipetted to the slide and sealed with a 18mm x 18mm coverslip. Rubber cement was applied to the coverslip to seal in the probe. Slides were then placed in a humid chamber at 37°C and allowed to hybridize for a minimum of 18 hours.

4.3.11 Fluorescence *in-situ* Hybridization - Post-hybridization Counterstaining

The following day, in a low light environment, the rubber cement and coverslip were carefully removed. Slides were placed in 0.4X SSC / 0.3% nonidet-40 (NP-40) for 1.5 minutes at 72°C to remove non-specific probe binding. Slides were then rinsed in 0.4X SSC / 0.3% NP-40 at room temperature for 2 minutes, then distilled water for 2 minutes to remove salts from the slide and air dried. 7µl of 4,6-diamidino-2-phenylindole

(DAPI II) counterstain was applied to the slide and coverslipped. DAPI II binds to A-T rich regions of the minor groove of DNA and fluoresces blue when exposed to ultraviolet light allowing visualization of the nucleus, which would otherwise be transparent under a fluorescent microscope (Kapuscinski, 1995). Slides were sealed with enamel and stored at –20°C until signal enumeration analysis.

4.4 Signal Enumeration

An Olympus BX51 fluorescence microscope was used for signal enumeration at 1500X under oil immersion. Cells must fall within specific criteria to be assessed. These criteria are based on the manufacturer's specifications:

1. Cells cannot overlap.
2. Nuclear membranes must be intact. Intact membranes have sharp, distinct edges whereas degraded membranes appear diffuse or blurry at the edges.
3. Non-specific binding, characterized by low intensity signals and different shapes, were not considered.

Cells that met the above criteria were assessed for the number of green centromere signals and red gene signals. A separate set of criteria for signal enumeration was used since signals may appear slightly different between patient samples.

1. Signal splitting, characterized by 2 high intensity signals in close proximity and joined together by a thin fluorescent strand, were considered a single signal.

2. Separate signals in close proximity that were not joined by a fluorescent strand with the same intensity were considered 2 signals.

Slides were taken from storage at –20°C and allowed to thaw to room temperature in the dark. Under oil immersion, the slide was focused and positioned to the upper left corner of the circle of cells. Enumeration of cells began from the top left to top right. Cells within the field of view and met the criteria described above were counted. When the slide reached the top right corner, the field of view was scrolled down and enumeration continued towards the left. The pattern is shown in Figure 7.

During the scoring process, a normal signal has 2 gene (g) and 2 chromosome (c) copies (2g2c). 400 to 500 normal cells were counted for each probe unless there were insufficient numbers of cells. In such cases, results were included if the total number of cells scored was approximately 400. Several patient samples from the ADC group did not contain adequate amounts of cells to carry out FISH procedures on both *3p14.2* and *9p21* molecular probes. Figure 8 is a photograph of FISH processed cells under a fluorescent microscope that shows how cells were enumerated based on the fluorescent signal. Photograph A shows a normal cell (g2c2) for *3p14.2* (green) and *3p* (red). Photograph B shows a g1c2 cell where one copy of the gene has been deleted.

4.5 Statistics and Analysis

Signal enumeration results were collected, and the raw data is shown in the appendix section. Comparison of demographic data (Section 5.1) was conducted using a t-test to compare two means.

The unmodified values for signal patterns were converted to percentages of the total cell count. A statistical analysis of specific signal patterns was conducted with SAS statistical program using a t-test or ANOVA for percentages. Percent values were converted because a percentage value does not indicate the sample size used and is not normally distributed. The modified t-test for proportions was used in analysis of all specific signal patterns and ANOVA was used for grouped signals (Section 5.4)

Analysis of grouped signal patterns (Section 5.5.1 to 5.5.3) were analysed using a modified t-test for percentages and a modified Dunnett's test, known as the Student-Neuman-Keul's (SNK) test for percentages (Section 5.5.2). Prior to comparison of the data, cut-off values were determined by taking the average percentages of signals plus two units of standard deviation from the ADC non-smokers and subtracted from current and former smokers from the ADC and the PCC group. The SNK procedure was used to compare grouped proportions of chromosome and gene abnormality rather than specific signal patterns. The SNK procedure compares the proportions of signals by converting the values using the following formula in radians:

$$\arcsin(\sqrt{p})$$

and employs the following formula to determine significance to P=0.05:

$$q_{(0.05)}(\kappa,\nu) \times \sqrt{(1/4n)}$$

where "p" is the proportion, "κ" is the number of comparison means, "ν" denotes the degrees of freedom (which is infinity in proportion comparisons), "n" is the sample size and "q" is the critical value on a Studentized distribution range. The numerical limit

required for significance in the SNK procedure is independent from the proportional data and dependent on the sample size only.

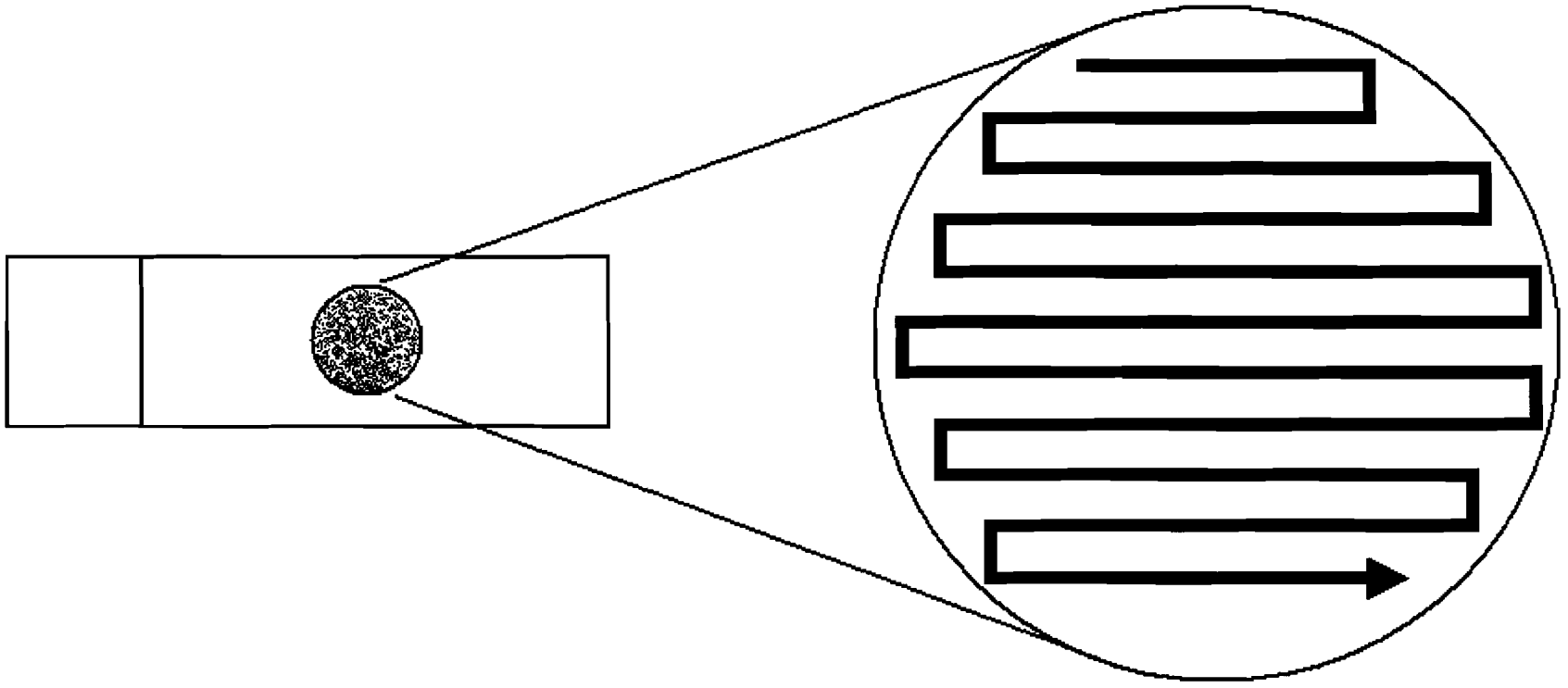


Figure 7 Cell scoring procedure on a FISH labelled slide

Exfoliated cells collected from the ventral-lateral tongue were adhered to a glass slide. After FISH processing, cells were examined on a fluorescent microscope and scored based on the criteria outlined in section 4.4. Scoring began on the top left area of the circle of cells and progressed horizontally to the border of cells on the right side. The microscope field of view was lowered and scoring progressed horizontally to the left until 400 normal cells were scored or the bottom of the circle of cells was reached.

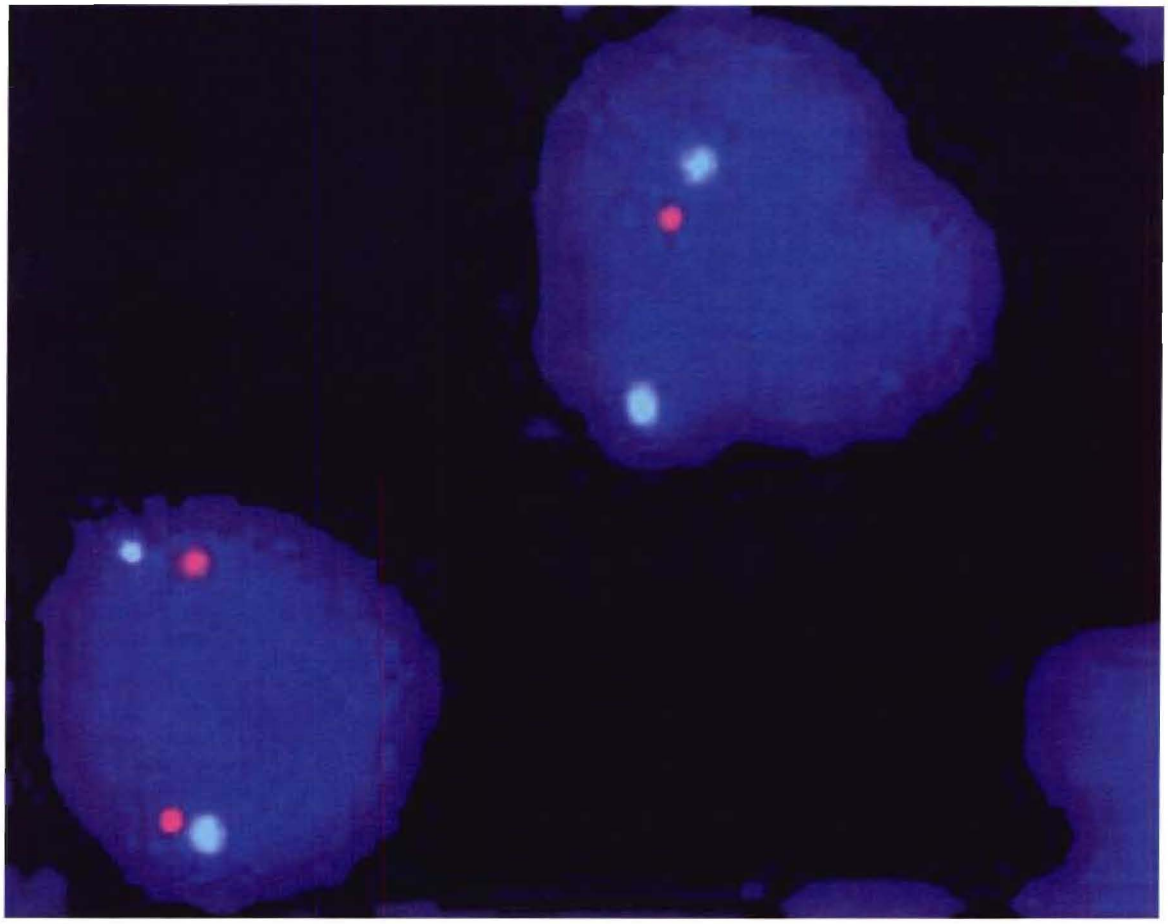


Figure 8 FISH signal pattern enumeration

Two cells counterstained with DAPI II gives the nuclear membrane a blue colour when visualized under a fluorescence microscope. The *3p14.2* FISH probe labels the gene region red and the corresponding CEP 3 chromosome probe labels the centromeric regions green. As shown above, the left cell exhibits a normal fluorescent signal of 2g2c (two copies of both the *3p14.2* gene and chromosome 3). The top right cell has lost a copy of the *3p14.2* gene while retaining both chromosomes, giving it a signal of 1g2c.

5. RESULTS

5.1 Demographic Data

Appendices 7.5 and 7.6 show the demographics, smoking and drinking data for both ADC and PCC groups. Table 2 shows the demographic data summary and comparison between the two groups. An t-test was conducted on the two groups.

Table 2 Comparison of demographic data between ADC and PCC patients

		ADC	PCC	P-value
Total	# Patients	51	53	
Gender	% Male	45	74	
Age	Mean Years Std. Dev. Median Range	51.6 10.8 53 30-80	51.1 9.8 52 35-76	0.79
Tobacco use	Former Smoker % Current Smoker % Non Smoker %	21.6 23.5 54.9	5.7 81.1 13.2	
Tobacco use among smokers only	Mean Pack-years S.D. Pack-years Range Pack-years	29.6 19.2 1.5-50.8	22.7 20.5 0.1-92.5	0.17
Tobacco use among all patients	Mean Pack-years S.D. Pack-years Range Pack-years	13.9 19.8 0-50.6	19.7 20.6 0-92.5	0.15
Alcohol use	Consumed alcohol more than 3 times annually (%)	63	85	

The ADC group included 51 patients with an average age of 51.6 years with 23 males and 28 females in the study. 28 were non-smokers, 12 were current smokers and 11 were former smokers. The average cigarette consumption among the entire group was

13.9 pack-years and increased to 29.6 pack-years when considering only current and former smokers. 32 of 51 (63%) patients consumed alcohol more than 3 times annually.

The PCC group included 53 patients with an average age of 51.1 years with 39 males and 14 females in the study. The high rate of males was due to the sampling site, which generally has more male residents. 7 were non-smokers, 43 were either current smokers and 3 were former smokers. The average cigarette consumption among the entire group was 19.7 pack-years and increased to 22.7 pack-years when considering only the current and former smokers. 45 of 53 (85%) patients consumed alcohol more than 3 times annually.

There was no significant difference between the age structures of the two populations ($p = 0.79$). The PCC group had a much larger proportion of male patients compared with the ADC group because the specific area where the PCC is located within the DTES is primarily inhabited by males based on verbal referral by Dr. Sean Sikorski the PCC dental clinician.

There was no significant difference in tobacco consumption between both groups ($p = 0.15$). It is interesting to note that when considering only smokers, ADC patients had a greater average tobacco consumption of 29.6 pack-years compared to the PCC values of 22.7 pack-years. The affordability of cigarettes among the ADC, middle-income patients and the availability of alternative drugs such as marijuana, cocaine, and crystal meth to the PCC group may be the cause of the relatively lower consumption rate in the PCC group. Smoking patterns also differed between the two groups. PCC smokers often reported very sporadic smoking patterns and an entire cigarette was less frequently smoked; rather, cigarettes often were passed around and used for a single puff while

being shared among multiple users throughout the day. This complicates the assessment of total pack-years consumed in the PCC population. Cigarettes are expensive relative to other drugs available in the DTES and some patients have shown a preference for other smokable drugs such as cannabis or crack cocaine. When all patients in both groups are considered, average tobacco consumption drops considerably to 13.9 pack-years in ADC, due to the high percentage of non-smokers (54.9%) compared to the 5.7% of non-smokers in the PCC. Alcohol consumption was also significantly different between the ADC and PCC groups at 63% and 85%, respectively. However, drinking patterns vary considerably and the incidence of alcohol addiction is assumed to be greater in the PCC.

Patient compliance is the rate of patients entering the dental clinic who complete both the questionnaire and the cell sample collection compared to the total number of patients that visited the clinic. Compliance is vital to any screening program, as a lengthy or uncomfortable examination may deter patients from participating. Factors such as the time required, invasiveness, comfort and the type of tests conducted all play a role in whether patients perceive the screening as beneficial to their health and hence, their willingness to participate. During the course of the study, the compliance rate was 87%. Those who began the screening process but did not complete the entire examination were considered non-compliant. Thus, the oral cytobrush technique was an effective method for screening as the procedure itself did not deter patients.

5.2 FISH Signals in ADC Patients

Appendices 7.7 and 7.8 show signal patterns represented in percentages for the *FHIT* locus *3p14.2* and *9p21*, respectively. Table 3 shows the summary data on the percentage of cells with specific signal patterns for *3p14.2* and *9p21* in ADC patients. The average number of cells with normal signals for *3p14.2* and *9p21* was 95.2% and 97.2%, respectively.

Table 3 Percentage of cells with specific FISH signal patterns for *3p14.2* and *9p21* in ADC patients

	% of cells with *3p14.2* signal	% patients with *3p14.2* signal	% of cells with *9p21* signal	% patients with *9p21* signal
# of patients	43		49	
Mean # of cells scored	462		413	
% of signals				
(normal) g2c2	95.2	100	97.2	100
g1c1	0.3	81	0.4	76
g2c1	1.1	100	0.3	71
g3c1	0.1	23	0	0
g0c2	0	5	0.2	41
g1c2	1.1	93	1.1	98
g3c2	1.1	100	0.2	45
g4c2	0.3	79	0.1	18
g2c3	0.3	70	0.1	31
g3c3	0.3	77	0.1	33
g4c4	0	12	0.2	39

As discussed above, the loss of both copies of either *3p14.2* or *9p21* in the genome allows cells to bypass critical regulatory steps of the cell cycle. In addition, a chromosome cannot exist without a centromere, therefore, alterations to centromere

numbers represents equal changes in chromosome numbers, or aneuploidy. However, our FISH procedures labelled only one chromosome at a time rather than entire sets of chromosomes, therefore alterations are termed monosomy (g=1) or polysomy (g>2).

Appendices 7.9 and 7.10 show five specific pattern types for comparison, termed Group A to Group E. Group A included signals with 2 gene copies but abnormal numbers of chromosomes. Group B included signals with abnormal gene copies with 2 chromosomes. Group C included all signals with abnormal numbers of chromosomes. Group D included all signals with abnormal gene copies. Group E included signals with abnormal numbers of genes and chromosomes. Table 4 shows a summary of the grouped aneuploidy data for *3p14.2* and *9p21* in the ADC group.

Table 4 Percent of cells with chromosome and *3p14.2* and *9p21* alterations in ADC patients

	% of cells with signal for *3p/3p14.2*	% of cells with signal for *9p/9p21*
# of patients	43	49
Mean # of cells scored	462	413
Group A[a] - Chromosome alterations only	1.5	0.5
Group B[b] - Gene alterations only	2.5	1.5
Group C[c] - All chromosome alterations	2.3	1.2
Group D[d] - All gene alterations	3.3	2.5
Group E[e] - Alterations in both gene and chromosome	0.8	0.8

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g2c3, g3c3, g4c3, g2c4, g4c4

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g3c3, g4c3, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g3c3, g4c3, g4c4

5.3 FISH Signals in PCC Patients

FISH results for PCC patients are shown in Appendices 7.11 and 7.12 for *3p14.2* and *9p21*, respectively. Table 5 shows the summarized results.

Table 5 Percentage of cells with specific FISH signal patterns for *3p14.2* and *9p21* in PCC patients

	% of cells with *3p14.2* signal	% patients with *3p14.2* signal	% of cells with *9p21* signal	% patients with *9p21* signal
# of patients	49		51	
Mean # of cells scored	439		443	
% of signals				
(normal) g2c2	91.2	100	90.6	100
g1c0	0.3	36	0	8
g2c0	0.9	68	0.1	8
g0c1	0	2	0.1	10
g1c1	1.5	92	0.9	69
g2c1	2.9	100	0.2	40
g0c2	0	8	2.6	96
g1c2	1.3	94	3.3	98
g3c2	1.4	80	0.4	67
g4c2	0.1	26	0	8
g1c3	0	0	0.1	25
g2c3	0.1	24	1.1	77
g3c3	0.2	38	0.2	46
g2c4	0	2	0.3	31
g4c4	0.1	20	0.1	35

Appendices 7.13 and 7.14 show the groups of chromosome and gene alterations for signal pattern groups A through E as defined previously, for *3p14.2* and *9p21*, respectively. Table 6 shows a summary of the grouped monosomy/polysomy data for *3p14.2* and *9p21* in the PCC group

Table 6 Percent of cells with chromosome and *3p14.2* and *9p21* alterations in PCC patients

	% of cells with signal for *3p/3p14.2*	% of cells with signal for *9p/9p21*
# of patients	50	52
Mean # of cells scored	439	443
Group A[a] - Chromosome alterations only	3.9	1.7
Group B[b] - Gene alterations only	2.8	6.2
Group C[c] - All chromosome alterations	6	3.2
Group D[d] - All gene alterations	4.9	7.7
Group E[e] - Alterations in both gene and chromosome	2.1	1.5

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g2c3, g3c3, g4c3, g2c4, g4c4

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g3c3, g4c3, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g3c3, g4c3, g4c4

5.4 Comparison of FISH Signals in ADC and PCC Patients

Table 7 is a comparison of FISH signals between ADC and PCC patients. A modified t-test for proportions was conducted to test for significance.

Table 7 Comparison of FISH signals in *3p14.2* between ADC and PCC patients

	ADC	PCC	
	% of cells with *3p14.2* signal	% of cells with *3p14.2* signal	P Value
# of patients	43	49	
Mean # of cells scored	462	439	
% of signals			
(normal) g2c2	95.2	91.2	0.005
g1c0	0	0.3	0.009
g2c0	0	0.9	0.004
g1c1	0.3	1.5	0.006
g2c1	1.1	2.9	0.005
g3c1	0.1	0	0.007
g1c2	1.1	1.3	0.17
g3c2	1.1	1.4	0.31
g4c2	0.3	0.1	0.005
g2c3	0.3	0.1	0.005
g3c3	0.3	0.2	0.13
g4c4	0	0.1	0.24

The comparison shows a significant difference between the proportion of normal cells. The signal patterns that were significantly higher in the PCC population were g1c0, g2c0, g1c1, g2c1, g4c2 and g2c3. All patterns except g4c2 show changes in centromere numbers with only g3c1 showing slightly higher signal rates in the ADC group.

Table 8 shows the same comparison between ADC and PCC patients for *9p21*. A modified t-test for proportions was conducted to test for significance.

Table 8 Comparison of FISH signals in *9p21* between ADC and PCC patients

	ADC	PCC	
	% of cells with *9p21* signal	% of cells with *9p21* signal	P Value
# of patients	49	51	
Mean # of cells scored	413	443	
% of signals			
(normal) g2c2	97.2	90.6	0.003
g2c0	0	0.1	0.08
g0c1	0	0.1	0.15
g1c1	0.4	0.9	0.04
g2c1	0.3	0.2	0.07
g0c2	0.2	2.6	0.002
g1c2	1.1	3.3	0.004
g3c2	0.2	0.4	0.09
g4c2	0.1	0	0.42
g1c3	0	0.1	0.009
g2c3	0.1	1.1	0.006
g3c3	0.1	0.2	0.03
g2c4	0	0.3	0.002
g4c4	0.2	0.1	0.16

A data summary comparison is shown in Table 9. A modified t-test for proportions was conducted to test for significance. The average percent of abnormal cells in groups A through E in *3p/3p14.2* and *9p/9p21*were consistently higher in the PCC group. An ANOVA test showed statistically significant differences between various ADC and PCC groups. Interestingly, for *3p14.2*, chromosome alterations increased significantly by 2.6-fold for both groups A and C. Gene alterations for the same comparisons increased by only 1.1-fold and 1.5-fold for groups B and D. The fragile site

associated with *3p14.2* may play a role in greater degree of significance in groups A, C and E, patterns that include chromosome number alterations. Gene alteration only comparisons in group B were not significantly different, but were when all gene alterations were considered in Group D.

The opposite pattern was seen with *9p21* comparisons. Although ADC and PCC risk comparisons between groups A and D were all significantly different (p-value < 0.001), gene alterations are more frequent than chromosome alterations. Gene alterations increased by 4.1-fold and 3.1-fold in groups B and D compared to chromosome alteration increases by 3.8 and 2.6 times for groups A and C.

Table 9 ANOVA comparison of specific FISH patterns between patient groups

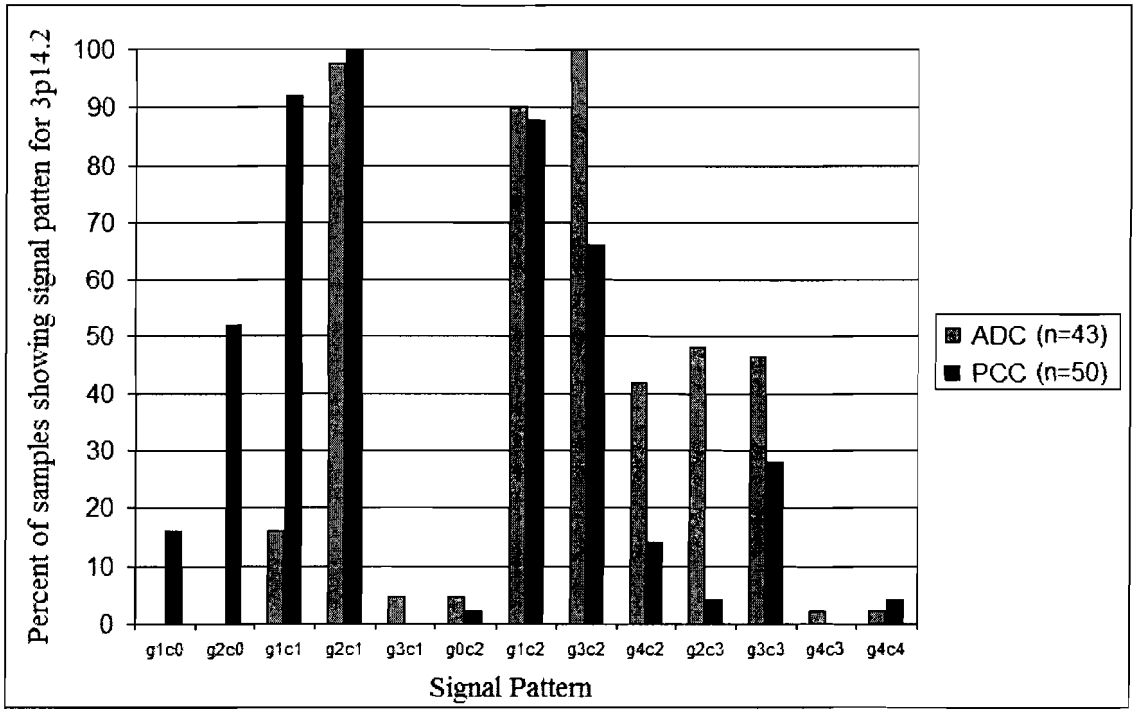
	Group A: Chromosome alterations only	Group B: Gene alterations only	Group C: All chromosome alterations	Group D: All gene alterations	Group E: Alterations in both gene and chromosome
ADC *3p14.2*	1.5	2.5	2.3	3.3	0.8
PCC *3p14.2*	3.9	2.8	6	4.9	2.1
P-value	<0.001	0.25	<0.001	<0.001	<0.001
ADC *9p21*	0.5	1.5	1.2	2.5	0.8
PCC *9p21*	1.7	6.2	3.2	7.7	1.5
P-value	<0.001	<0.001	<0.001	<0.001	0.017

Figure 9A and Figure 9B compares the percentage of samples, which show the presence of any particular abnormal pattern in order to determine signal patterns that occur most frequently, but not considering the number of abnormal cells for each signal. This provides a quick reference to the prevalence of specific patterns within patients. Figure 10A and Figure 10B shows the percentage of abnormal cells in each group for their respective FISH probes. Although the prevalence of a signal may be high based on

the percentage of patients who exhibit the pattern shown in Figure 10, this may be misleading if relatively few cells are responsible for its detection.

Figure 9 Proportion of samples that exhibited abnormal FISH patterns in *3p14.2* (A) and *9p21* (B)

A) *3p14.2*



B) *9p21*

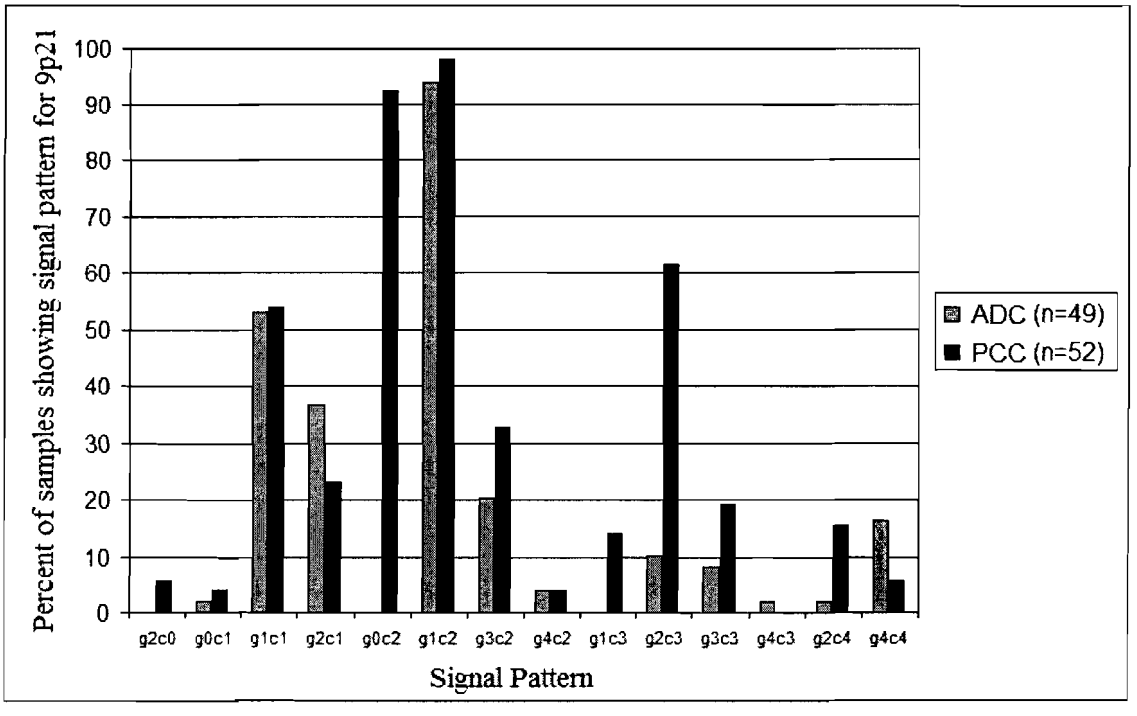
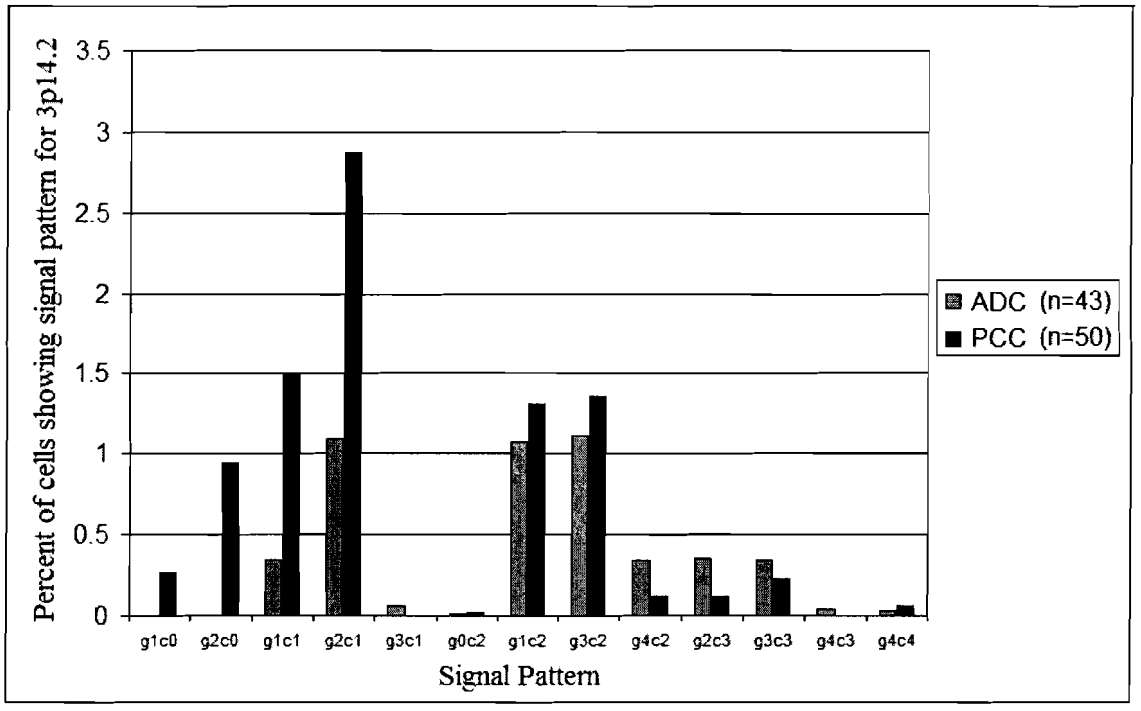
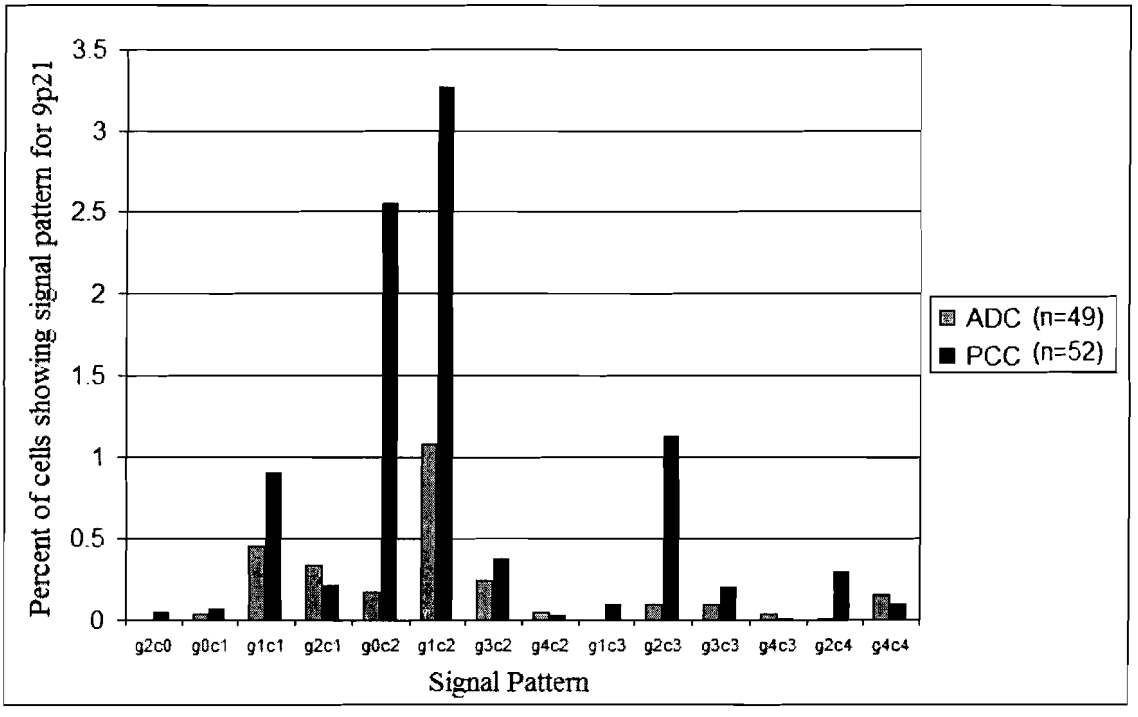


Figure 10 Proportion of cells that exhibited abnormal FISH patterns in *3p14.2* (A) and *9p21* (B)

A) *3p14.2*



B) *9p21*



5.5 FISH Signal Analysis

To determine whether specific FISH patterns are associated with the PCC group, and therefore with an elevated risk of OSCC, statistical analysis was conducted in two ways. First, abnormal signals were identified based on the number of patients possessing specific patterns regardless of the number of cells found. Characterizing the incidence of abnormal signals will narrow the possible candidate signals associated with risk. Secondly, signals were also identified based on the percentage of abnormal cells in a patient sample.

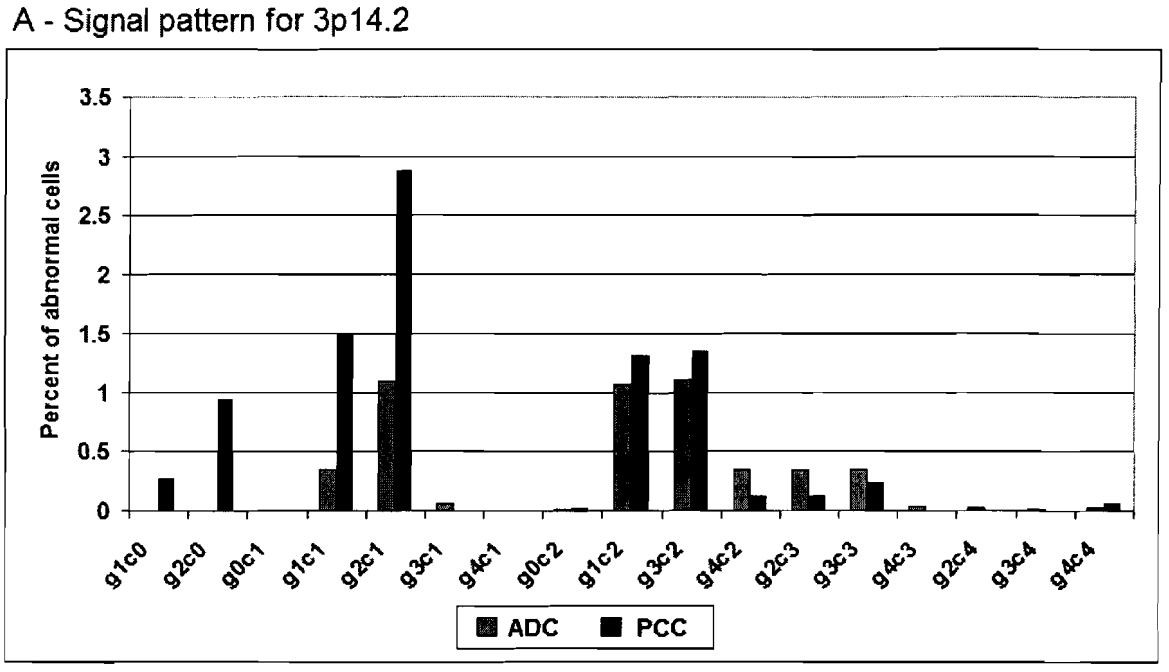
With both methods, cut-off values were determined with ADC non-smokers as a reference and compared to ADC smokers/former smokers and PCC patients using a box-plot analysis. Cut-off values were set as the average plus 2 units of standard deviation. An ANOVA test was used to compare FISH patterns between groups, and a Student-Newman-Keuls test (SNK) was used to compare proportions between groups.

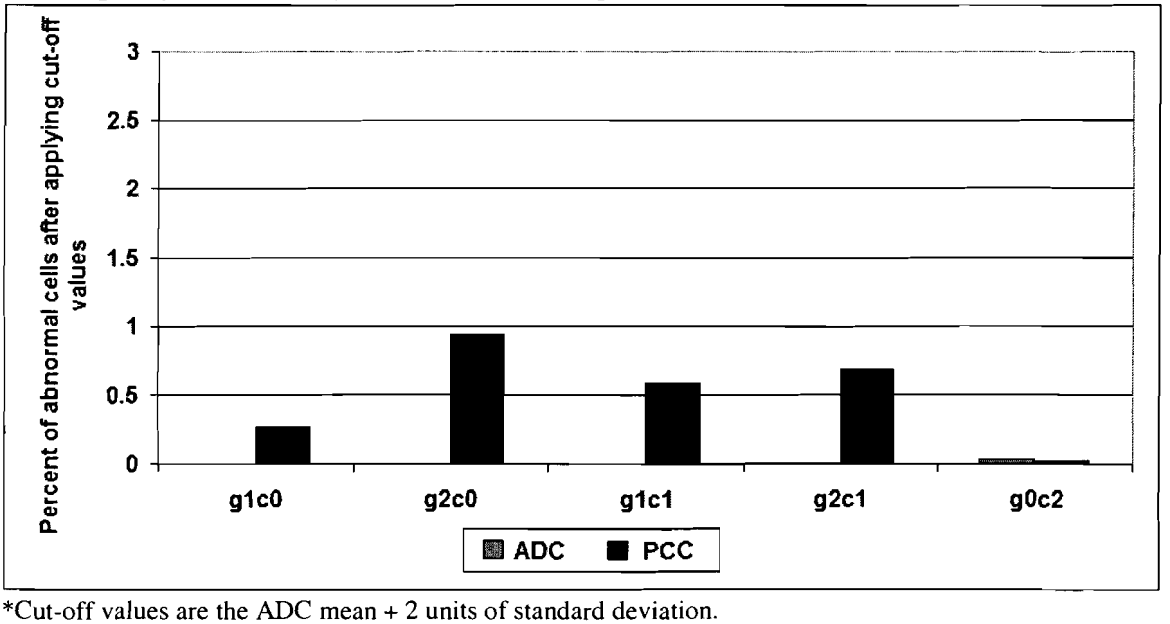
The cut-off values were set as the ADC non-smoker mean plus 2 standard deviation units. Figure 11A represents the average signal percentages for *3p14.2* from ADC and PCC groups from Table 3 and Table 5. Figure 11B shows the same data after cut-off values were implemented. Signal patterns: g1c0, g2c0, g1c1 and g2c1 in high-risk patients were the only signals that exceeded the cut-off values and tested significantly different from Table 7.

Figure 12A shows the same comparison for *9p21*, which the summary data is shown in Table 3 and Table 5. Figure 12B shows the same data after implementing cut-off values. Although a greater number of patterns emerged compared to *3p14.2*, most

were not significantly different from ADC comparisons based on Table 8. Six patterns, g1c0, g0c2, g1c2, g1c3, g2c3 and g2c4 showed significant levels of abnormality.

Figure 11 Average percentage of abnormal cells for *3p14.2* before (A) and after (B) applying cut-off values.

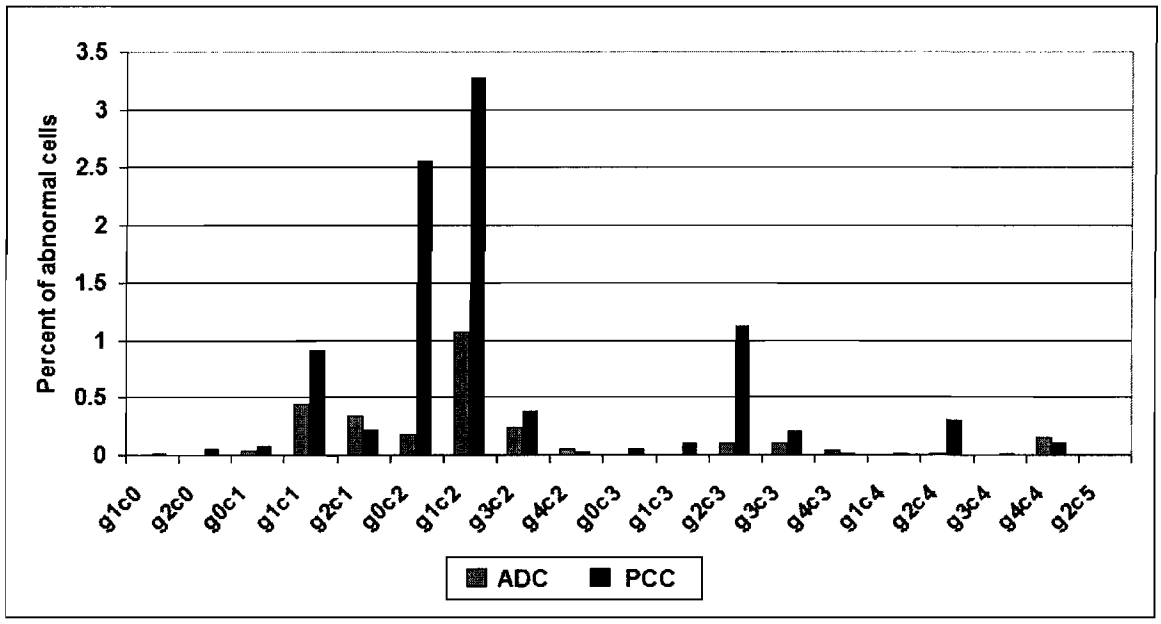




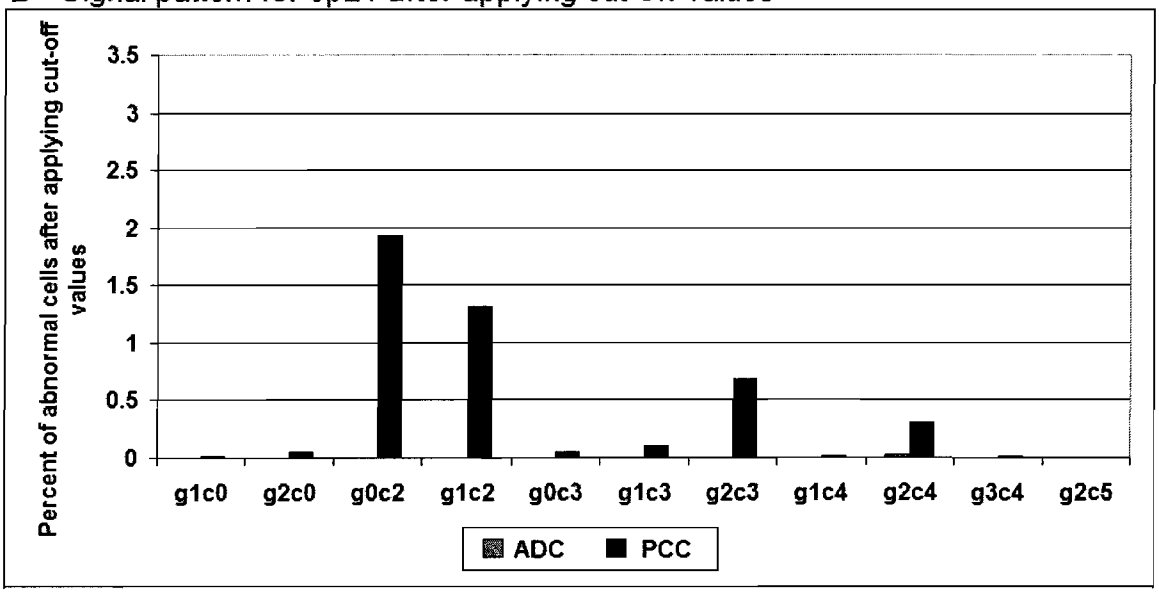
*Cut-off values are the ADC mean + 2 units of standard deviation.

Figure 12 Average percentage of abnormal cells for *9p21* before (A) and after (B) applying cut-off values.

A - Signal pattern for 9p21



B - Signal pattern for 9p21 after applying cut-off values



*Cut-off values are the ADC mean + 2 units of standard deviation.

5.5.1 Analysis of Patients with Abnormal Signal Patterns

Cut-off values were derived for grouped signal patterns from A to E shown in Table 10. Table 11 shows the number of ADC and PCC patients that exceeded the established cut-off values. For *3p14.2*, there was a significantly greater proportion of PCC patients that had signal patterns related to chromosome alterations (Groups A and C) compared to gene alterations (Groups B and D). This was not found in the ADC group. In group A, considering only chromosome alterations, 60% of PCC patients exceeded cut-off values. In group C, which includes all chromosome alterations, this rate increased to 70%. This is compared to 28% of PCC patients in group B, which increases to 48% in group D, when considering gene alterations plus any chromosome pattern. Alterations in chromosome numbers appear to be more strongly associated with PCC individuals when comparing *3p14.2*.

For *9p21*, 90% of PCC patients exhibited abnormal gene patterns (group B), but only 44% had chromosome alterations. Alterations in *9p21* gene and chromosomes within a cell were less frequent than with *3p14.2* at only 13% compared to 38%, respectively. The data shows a trend of more frequent chromosome abnormalities associated with the *3p14.2* region compared to more frequent gene abnormalities associated with *9p21*.

Table 10 Cut-off values of FISH signal percentages for *3p14.2* and *9p21* from ADC non-smokers

		3p14.2	*9p21*
# of patients		28	28
Group A[a] - Chromosome alterations only	Mean	1.6	0.4
	SD	0.6	0.3
	Mean + 2SD	2.8	1.0
	Median	1.7	0.3
Group B[b] - Gene alterations only	Mean	2.2	1.2
	SD	0.7	0.9
	Mean + 2SD	3.7	2.9
	Median	2.4	0.9
Group C[c] - All chromosome alterations	Mean	2.3	1.1
	SD	0.9	0.8
	Mean + 2SD	4.1	2.6
	Median	2.2	0.7
Group D[d] - All gene alterations	Mean	3	1.9
	SD	1	1.3
	Mean + 2SD	5.1	4.5
	Median	3.0	1.5
Group E[e] - Alterations in both gene and chromosome	Mean	0.7	0.7
	SD	0.5	0.6
	Mean + 2SD	1.8	1.9
	Median	0.6	0.6

* Cut-off values set as the average + 2 standard deviation units

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g2c3, g3c3, g4c3, g2c4, g4c4

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g3c3, g4c3, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g3c3, g4c3, g4c4

Table 11 Comparison of patients (%) with FISH signal patterns that exceeded cut-off values established using ADC non-smoker means + 2 units of standard deviation

	ADC current and former smokers	PCC	P value
3p14.2			
Total # of patients	23	50	
Group A - Chromosome alterations only	0 (0%)	30 (60%)	0.004
Group B - Gene alterations only	1 (4%)	14 (28%)	0.005
Group C - All chromosome alterations	1 (4%)	35 (70%)	0.002
Group D - All gene alterations	3 (13%)	24 (48%)	0.007
Group E - Alterations in both gene and chromosome	4 (17%)	19 (38%)	0.009
9p21			
Total # of patients	23	53	
Group A - Chromosome alterations only	4 (17%)	23 (44%)	0.009
Group B - Gene alterations only	5 (22%)	47 (90%)	0.009
Group C - All chromosome alterations	2 (9%)	19 (36%)	0.008
Group D - All gene alterations	5 (22%)	42 (81%)	0.006
Group E - Alterations in both gene and chromosome	3 (13%)	7 (13%)	0.74

5.5.2 Analysis of Abnormal Signal Pattern Rates

To determine if a detectable level of significance exists between the ADC and PCC groups, ANOVA was used to analyse signal patterns after applying cut-off values. The Student-Newman-Keuls (SNK) test was used to compare proportions of signal patterns between groups. The SNK procedure was used to compare the chromosome and gene abnormality groups and not individual signal patterns. It is difficult to detect significance using this method based on the small degree of differences in proportions between specific signals and the sample sizes used in this study. Using the SNK formula to calculate in reverse, an approximate sample size of 200 patients per risk group would be

required to detect significant differences in signal patterns. Therefore, the maximum critical limit of 0.21 for a sample size of 43 was the same for all group comparisons within *3p14.2*. In *9p21* comparisons, the maximum critical value was 0.20 for a sample size of 49. Table 12 shows the critical values required for significance between comparisons using the SNK procedure. None of the comparisons showed any significance differences.

Table 12 Minimum critical value of the Student-Newman-Keuls test allowable for significance of 0.05 comparing ADC and PCC

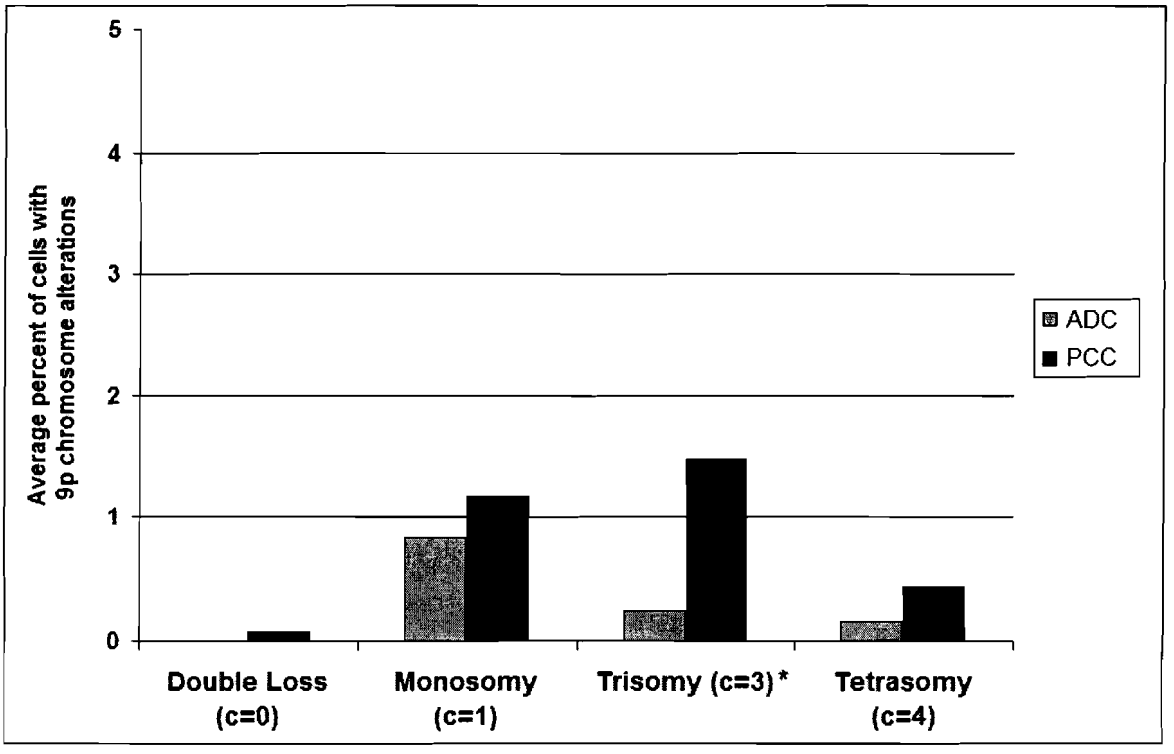
	Minimum critical value of SNK test
3p14.2*	
Total # of patients	43
Group A - Chromosome alterations only	0.13
Group B - Gene alterations only	0.05
Group C - All chromosome alterations	0.15
Group D - All gene alterations	0.07
Group E - Alterations in both gene and chromosome	0.07
9p21	
Total # of patients	49
Group A - Chromosome alterations only	0.08
Group B - Gene alterations only	0.15
Group C - All chromosome alterations	0.09
Group D - All gene alterations	0.1
Group E - Alterations in both gene and chromosome	0.05

* A difference of 0.21 or higher is required for significance with a sample size of 43.
** A difference of 0.20 or higher is required for significance with a sample size of 49.

5.5.3 Analysis of Polysomy, Monosomy and Double Centromere Loss

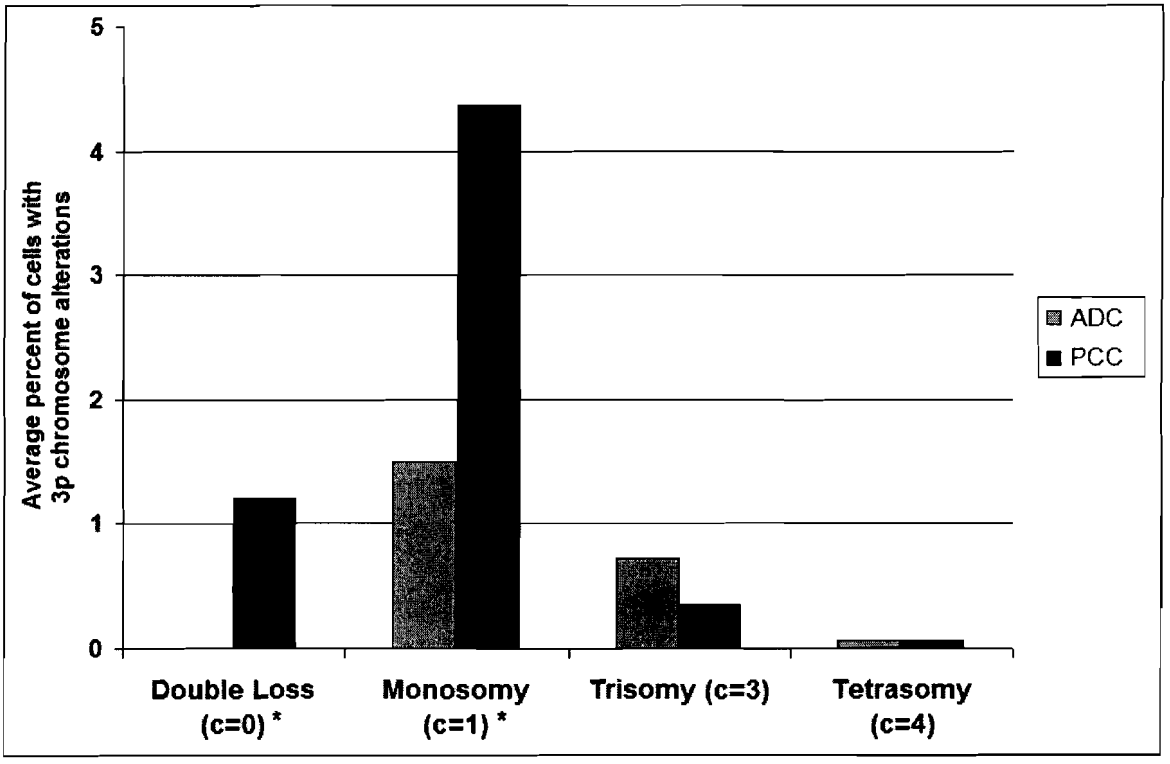
Figure 13 and Figure 14 show the incidence of double centromere loss, monosomy and polysomy for *9p* and *3p*, respectively. A modified t-test for proportions was conducted to test for significance. *9p* shows a significant increase of trisomy in PCC patients, with no other signals showing significance. *3p* in PCC patients shows significant increases in double centromere loss and monosomy, but no differences in trisomy or tetrasomy. The difference in patterns can likely be attributed to the presence of the fragile site in the *3p14.2* arm and its potential effects on chromosome instability which is not present in *9p*.

Figure 13 Incidences of *9p* chromosome loss or gain



* Significantly different ($P < 0.01$)

Figure 14 Incidences of *3p* chromosome loss or gain



* Significantly different

6. DISCUSSION

This study had four main objectives:

1.) To identify specific patterns of genetic change within *3p/3p14.2* and *9p/9p21* that are present in individuals with an elevated risk of OSCC development by comparison to a representative population with an average risk of OSCC.

2.) To provide patients at elevated risk of OSCC with free oral cancer screening.

3.) To determine the acceptability of the oral cytobrush procedure among dentists and patients.

4.) To determine the ease of integrating the oral cytobrush procedure and a health questionnaire into a regular dental visit.

For the first objective, by comparing the rate of abnormal signals within patients with the frequency of the patterns within the population, the signal patterns that were associated with the PCC population, from greatest association to least, were: g1c0, g2c0, g1c1 and g2c1 for *3p/3p14.2* and g0c2, g1c2, g2c3 and g2c4 for *9p/9p21* respectively. Grouped data showed that samples from PCC patients had a higher incidence of *3p* chromosome loss and *9p21* gene deletions.

The second objective was met from 2004 to the present (June 2007), and the study has examined over 400 DTES individuals for OSCC. The screening program is still running in the PCC as a related research project.

The third objective was assessed by examining the compliance rate of patients in the DTES. Overall, the rate of compliance, where patients completed the oral

questionnaire (Appendix 7.3) and were examined using the VELScope, toluidine blue test and the oral cytobrush technique was 85%. The most common reasons for non-compliance were time constraints, dental or oral pain which prevented examination or patients did not want to be disturbed. Overall, most patients regarded the procedure with positive feedback, reporting minimal discomfort, which was related to the acetic acid oral wash associated with toluidine blue staining. Dental clinicians responded positively to the procedure, expressing interest in learning OSCC screening practices due to its relative simplicity of the screening tools introduced to the clinic.

The fourth objective was assessed by examining the delay period that patients experienced as a result of participating in the study. The duration of the oral health screening, which included the questionnaire, VELScope examination, toluidine blue staining and oral cytobrush technique was 15 minutes per patient.

The questionnaire and physical testing was usually conducted prior to the scheduled dental appointment. Patients whose appointment began before the entire screening could be completed were asked to voluntarily remain after their appointment to complete the screening process. Approximately 15% of patients experienced this delay, which usually did not last more than 10 minutes.

3p14.2 and *9p21* were ideal TSG candidates for this investigation because they are common sites for mutational loss in the early stages of carcinogenesis (Huebner and Croce, 2003; Shintani *et al.*, 2001). Changes in centromere numbers, indicative of whole chromosome loss, gives rise to whole cell chromosome instability, thus increasing the susceptibility of the genome for further mutational change. The use of multiple TSGs or oncogene probes to simultaneously assess multiple TSG loss or oncogene amplifications

makes FISH a powerful for assessing genetic abnormalities that may lead to the development of OPLs. If validated, a fast assessment method such as FISH may increase the early detection rate of OPL and consequently reduce OSCC mortality rate. In regards to the FISH procedure itself, the processing time within the dental clinic is very fast. The oral cytobrush procedure requires less than 2 minutes to conduct. The most time-consuming portion of the procedure is the actual FISH procedure and cell enumeration in a laboratory setting. However, FISH still provides advantages over histological analysis of biopsy tissue because it does not require a trained pathologist to examine the tissue and the signal enumeration is not as subjective to interpretation as histology analysis.

Cell mutational rates for *3p* and *9p* centromere signals were 2.3% and 1.2% respectively in the ADC group. The minimum percentage of cells with abnormal centromere signals estimated to constitute a significant elevation in OSCC risk is 5% (Veltman *et al.*, 1997). For the gene loci *3p14.2* and *9p21*, mutation rates were 3.3% and 2.5%, respectively. Based on this criteria, the ADC is not considered at a significantly elevated risk for OSCC development; however, there has been no established mutation rate above which an elevated OSCC risk exists. PCC individuals had higher mutation rates for both *3p/3p14.2* and *9p/9p21* with rates of 6.0%/4.8% and 3.2%/7.7%, respectively. The *3p* centromere rate exceeded the 5% limit on average ($p<0.001$) though the *9p* did not.

6.1 FISH Patterns in *3p14.2*

The suggestion that centromeric alterations initiate chromosome instability in early carcinogenesis has been proposed in various studies (Duesberg, 2005; Duesberg *et al.*, 1998; Storchova and Pellman, 2004). In the present study, a large proportion of the

cells with abnormal signal patterns retain the gene copies in the absence of the centromere. The *FHIT* region is the most common site of familial translocations and the most common site of strand break in human chromosomes (Ohta *et al.*, 1996). Translocation processes may transfer part of the gene to an adjacent chromosome, resulting in a positive signal in FISH but ultimately leading to the loss of the centromere, producing signals where gene copies exceed chromosome copies. It is unclear whether this translocation would affect *3p14.2* gene product production in the cell, as currently no studies have attempted to quantify *3p14.2* gene products under these conditions. However, our results are in line with studies examining *3p14.2* protein expression, where reductions in *FHIT* protein expression was associated with an increased relative risk and an indicator of poor prognosis in OSCC and other cancers such as cervical cancer (Huang *et al.*, 2003; Mineta *et al.*, 2003). It should also be noted that the presence of g1c0 and g2c0 signals were found exclusively in the PCC group (Figure 9A), whereas single centromere losses such as g1c1 and g2c1 were found in both groups. Almost all patients in both the ADC and PCC groups had g2c1 signals, but the proportion of cells was greater in the PCC. Incidences of double centromere loss regardless of gene copies (g1c0 and g2c0) may indicate an elevated risk, with single centromere loss (g1c1 and g2c1) being an earlier mutational event. The latter, however, may occur too early to effectively assess OSCC risk, since signal patterns are found in both groups.

6.2 FISH Patterns in *9p21*

9p21 FISH patterns are considerably different from *3p14.2*. The lack of a fragile site in *9p21* may account for the lower frequency of centromere loss. Gene loss signals g0c2 and g1c2 occur most frequently in the PCC population accounting for

approximately 2.6% and 3.3% of all scored cells respectively. Two other patterns, g2c3 and g2c4 are not as common, and may represent early manifestations of replication problems and DNA segregation. The signals g1c0 and g1c3 occurred fairly infrequently among the PCC group and did not have very high rates of cell counts.

The deletion of *9p21* copies is clearly associated with PCC individuals shown on Table 11. g0c2 and g1c2 signals greatly exceed ADC rates with g0c2 occurring exclusively in the PCC shown in Figure 11B. For g1c2, both populations share the same proportions of individuals who had the abnormal signal, but cell count rates were much greater in the PCC (Figure 9B). Based on these results, g0c2 shows the greatest association with the DTES group followed by g1c2 with the possibility of centromere amplification (g2c3, g2c4) playing a role as well.

6.3 Biological Significance of Centromere 9 and *9p21* Numerical Alterations in OSCC and OSCC Risk

The genetic patterns that were found to be associated with *9p* and *9p21* in this study are consistent with current hypotheses on the genetic changes required for OSCC development. The current model for cancer development requires both copies of *9p21* gene to be deleted or inactivated before a cell loses the gene function. This study examines populations with elevated risk with no symptoms of OPL or OSCC, therefore, we expected to see a higher proportion of cells with single copies of numerical alterations, compared to homozygous deletion. The average percent of g0c2 and g1c2 cells in PCC were 2.6% and 3.3% relative to 0.2% and 1.1% in the ADC group.

The loss of $p16^{INK4a}$ and $p14^{ARF}$ protein products is found in over 80% of all head and neck SCC cases (Forastiere *et al.*, 2001). The high rate of 9p21 deletion in OSCC and

its susceptibility to early loss makes it an ideal TSG candidate to study. The genes that *9p21* codes for are critical to normal cell growth and proliferation. Their relative positions, overlapping but on alternative template DNA strands suggests they are highly conserved since mutations in the coding region will affect both gene products. It can also result in the simultaneous inactivation of both genes as a result of mutation, such as deletions, affecting both the G_1 checkpoint and *p53* activity, making *9p21* inactivation more detrimental than most other TSGs (Berggren *et al.*, 2003).

Tobacco related carcinogens which cause gene mutations are well documented, causing deletions, DNA adducts, double-stranded DNA breaks, formation of reactive oxygen species related to DNA damage and the development of aneuploidy (Ando *et al.*, 2003; DeMarini, 2004; Nelson *et al.*, 1998). Aneuploidy was initially believed to be an artifact resulting from cancer, but evidence now suggests that it is a precursor to cancer and can be used as a potential marker to indicate cancer risk (Fenech, 2002; Storchova and Pellman, 2004). Various hypotheses exist for the initial development of aneuploidy. One hypothesis suggests that aneuploidy can develop as a result of gene mutations in cell division processes such as mitotic spindle formation, causing mis-segregation of chromosomes and forming tetraploid cells (4n) (Storchova and Pellman, 2004). Subsequent cell replication, also containing mis-segregation of chromosomes leads to various forms of aneuploidy cells.

The scope of this study did not assess aneuploidy, for which entire chromosome sets must be quantified. Rather, single chromosomes were detected using FISH and therefore termed monosomy (c1) or polysomy (c>2). Based on previous research from our lab and current beliefs on oral cancer development, we have proposed that a sequence

of genetic and histological events leading to OSCC generally occur in the order described in Figure 15. Normal tissue is exposed to tobacco-related carcinogens resulting in early onset of *9p/9p21* deletions or subsequent inactivation, followed by changes in other TSGs or oncogenes leading to tetraploidy, aneuploidy and finally OSCC. As the proportion of affected cells increases over time, we expect to see single *9p21* copy deletions as the most common occurrence, followed by tetraploidy, various forms of aneuploidy (e.g. monosomy and trisomy), double gene copy deletions and finally OSCC. This proposed pathway is supported by the incidences of double centromere loss, monosomy, trisomy and tetrasomy shown in Figure 13.

An unexpected result from the analysis shows *9p21* deletions are common in the PCC group, but they were not accompanied by equal rates of centromere deletions. Rather, the amplification of *9p* signals suggests the early onset of genetic instability. *9p* polysomy has been associated with specific histological characteristics of leukoplakias using FISH with higher rates of *9p* polysomy related to high-risk OPLs (Kim *et al.*, 2001). That study found median rates of *9p* trisomy (c=3) in hyperplasia, hyperplasia/dysplasia and dysplasia samples to be 1.5%, 2.4% and 2.4% respectively (although no mean percentage was reported for comparison). This study found mean trisomy rates (g1c3, g2c3) at 0.8% shown in Figure 12B, which was expected since PCC patients had no incidence of any OPL.

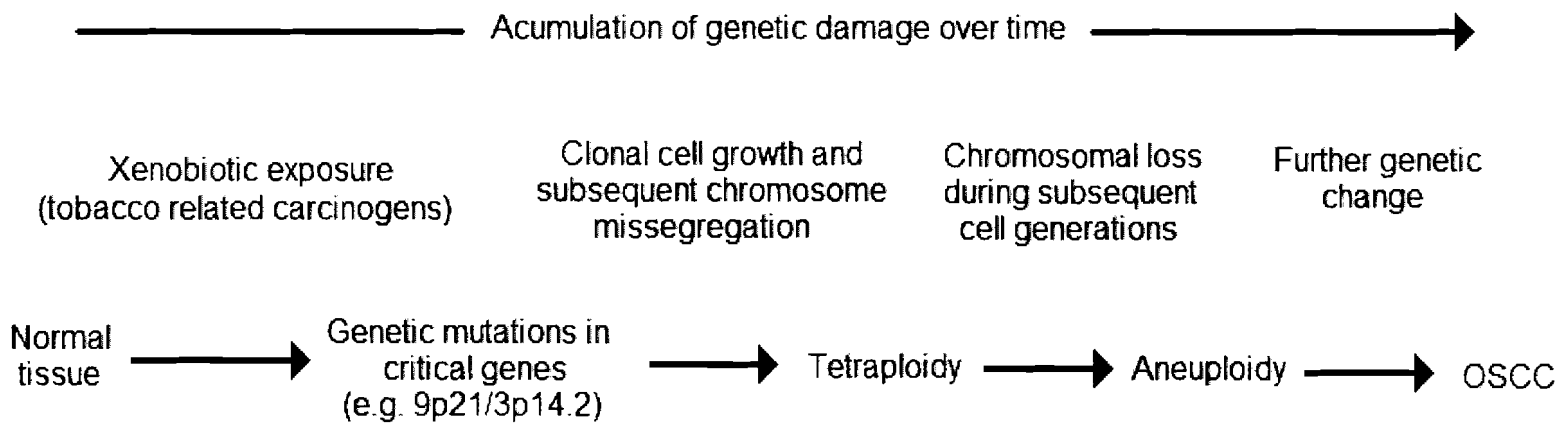


Figure 15 Proposed temporal sequence of genetic mutational events from normal cells leading to OSCC.

Adapted and modified from (Barnes, 2003)

6.4 Biological Significance of Centromere 3 and *3p14.2* Numerical Alterations in OSCC and OSCC Risk

3p/3p14.2 alterations in this study show chromosome loss is more frequent than gene deletion based on the signals associated with high-risk individuals (g1c0, g2c0, g1c1 and g2c1) from Figure 11B and comparisons of grouped centromere alterations in Table 9. The fragile site within the *3p14.2* arm of the chromosome is spatially distinct from the centromere. Gene deletions typically do not affect the centromere, but this may be untrue for fragile sites such as *FHIT*.

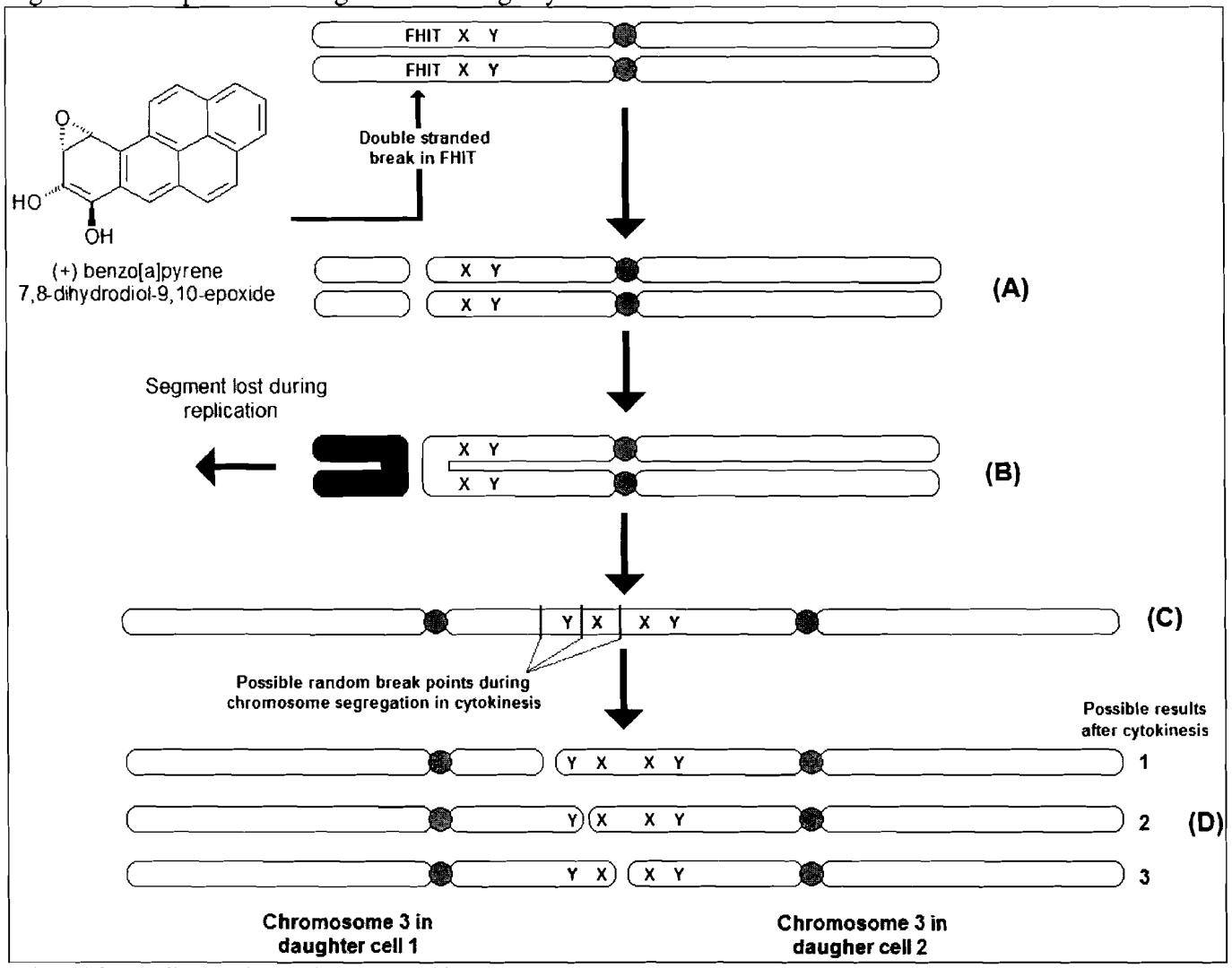
Studies suggest that xenobiotic-induced strand breaks at *FHIT* induces subsequent centromeric loss in a process described as a breakage-fusion-bridge cycle (Birnbaum *et al.*, 2003; Ciullo *et al.*, 2002; Popescu, 2003). BAP is the most researched tobacco-related carcinogen, which is metabolised to its ultimate carcinogenic form as shown in Figure 1. The BAP epoxide forms genetic lesions by covalently binding to guanine nucleotides and induces strand breaks, particularly in *FHIT*. Figure 16 shows the hypothesized mechanism involved in this process (Alberts, 1994; Fleming, 2002). DNA replication and repair mechanisms that fail to remove the lesion results in double stranded breaks. In the breakage-fusion-bridge cycle, the *FHIT* region breaks (A) and the adjacent ends fuse together (B), resulting in a chromosome with two centromeres and a DNA segment without a centromere. The latter is lost during replication due to mis-segregation from lack of a centromere. As cells undergo mitosis, spindle fibres attach at the centromere and pull the same chromosome to opposite ends of the daughter cells during cytokinesis and causing a random break in the DNA between the two centromeres (C). The resulting

daughter cells lack a copy of the centromere and possibly inactivate one or both copies of *FHIT* related genes (D).

The breakage-fusion-bridge cycle is proposed to be a precursor to chromosomal instability leading to subsequent mutational events in the *3p* chromosome among many other fragile sites in different chromosomes (Arlt *et al.*, 2006). The sequence also explains the presence of the signals g1c0, g2c0, g1c1, g2c1 where copies of *FHIT* exceed the number of centromere copies related to that chromosome. This is further supported in Figure 14 showing incidence of *3p* centromere alterations in this study with significant increases in monosomy and double centromere loss. Additional studies show microsatellite instabilities and loss of heterozygosity occurring before *FHIT* gene alterations (Boldog *et al.*, 1997) and colorectal cancers also support this hypothesis (Boland *et al.*, 1998; Sarli *et al.*, 2004; Thibodeau *et al.*, 1993).

Currently, there is little research implicating abnormal *3p/3p14.2* genetic patterns with specific losses in cell phenotype. There is little definitive research that examines the function and exact mechanism of *FHIT* related protein products. The most recent research suggests pathways regulating apoptosis at the DNA damage checkpoint (Ishii *et al.*, 2006; Sard *et al.*, 1999), and intracellular diadenosine triphosphate concentrations (Murphy *et al.*, 2000).

Figure 16 Proposed breakage-fusion-bridge cycle in *FHIT*



Adapted from Alberts, 1994 and Fleming, 2002

6.5 FISH as an Assessment Tool of OPL and OSCC Risk

The use of FISH for OPL or OSCC risk prediction is a new concept and is currently not established in a clinical setting. The loss of *9p21* and *3p14.2* is associated with OSCC patients in LOH studies but rates of abnormal cells constituting a risk has not been investigated.

ANOVA analysis of ADC and PCC groups had identified several abnormal signal patterns in *3p/3p14.2* and *9p/9p21* associated with the PCC group. None of the comparisons showed significance when a similar analysis was conducted using the SNK test for proportional data. It may not be biologically possible to detect the required proportion of abnormal cells in the brush field without cell death or even death of the individual. Hence, the differences seen may be biologically significant, because cancer originates as a clonal amplification of a single cell, but not statistically significant unless a higher sample size is used, which was beyond the time capacity of this study.

6.6 Limitations and Study Bias

Several study limitations and biases exist in this study.

1. Report of tobacco use is subject to recall bias
2. Lack of disease information for the Abacus Dental Centre
3. Lack of long-term follow-up of patients to examine outcome of severely high-risk individuals.

During data collection for both PCC and ADC patients, problems regarding accurate recollection of tobacco use became apparent. The survey used a single estimate

for weekly tobacco consumption and tobacco pack-year consumption was calculated based on the estimated average and the number of years the individual has smoked. This presented multiple problems.

First, individual smoking habits change during the lifetime. For example, many individuals experience periods of smoking cessation. The ability to afford cigarettes is also an issue, generally having lower cigarette consumption during teenage years with peak consumption during the ages of 20-30. Between the two groups, there is also a recall bias, where the accuracy of an individual's recollection of their past smoking habits may vary considerably, especially if the patient is much older. PCC patients generally had a poorer recollection of their past drug use than ADC patients. Also, smoking habits are extremely variable in the DTES area. Many PCC patients reported rarely smoking an entire cigarette, but rather, either took several puffs of a cigarette when it was offered among a group, or similarly, smoking the remaining tobacco from a discarded cigarette butt. The difficulty of accurately assessing total tobacco consumption with the DTES population is amplified by poor recollection of previous drug use and the occasional refusal of PCC patients to answer questions relating to past drug use for fear the information might be used by police or authorities.

Initial attempts to estimate tobacco consumption more accurately by modifying the questionnaire to identify precise smoking habits at specific age ranges (e.g.: <18, 19-24, 25-34 years) was met with additional problems. The additional time required to answer the questions placed constraints on time, which sometimes resulted in upsetting the patient, refusal to complete the questionnaire, refusal to continue with the oral brushing procedure, or replying to the questions with the exact same answer in an attempt

to expedite the survey resulting in decreased accuracy. As a result, the original questionnaire was retained to give a single estimate of tobacco consumption.

Alcohol consumption quantification experienced many of the same recall biases and use habits as tobacco. Notably, ADC patients were likely to have steady and predictable social drinking patterns and were less likely to have alcohol addiction and abuse relative to PCC patients. In comparison, PCC patients reported high incidences of binge drinking where alcohol consumption rates were temporarily very high. Patterns of drinking were also highly susceptible to the current financial status. Recollection of drinking rates and frequency would also be subject to recall bias in this situation because high alcohol consumption can impair memory. As a result, consumption was assessed as a positive or negative if the individual consumed more than 3 alcoholic beverages annually. Information on specific type of alcohol consumed and the estimated average weekly or monthly consumption was obtained, but the degree of variability among individuals answers made it impossible to accurately report.

Appendix 7.4 shows the reported incidences of hepatitis B, C, tuberculosis and HIV infection in the PCC patients. Similar data was not obtained for the ADC patients. The questionnaire addressed questions for multiple studies independent of this one, and disease incidence was not an issue until the study was brought to the DTES. As a result, there is no comparison for disease incidence between the two groups, but the data for the PCC is reported. There is no doubt that the DTES population has a greater incidence of these diseases, among others, compared to an average risk group such as the ADC patients.

Another limitation of the study is the lack of long-term follow-up of the patients. During the course of over 2 years, many PCC patients involved in the study were re-examined in subsequent dental visits. During this study, the VELScope and toluidine blue screening procedures were conducted in PCC patients. Patients that had any indications of increased risk, such as high toluidine blue staining, elevated risk based on VELScope visualization or high tobacco and alcohol consumption rates during their initial visit were re-examined if their follow-up dental appointment was at least 6 months later. However, oral mucosa cell samples were not obtained in follow-up examinations. The data collected on follow-up visits are intended for related research projects but not addressed here. The time required to conduct long-term follow-up analysis greatly exceeds the scope of this study.

6.7 Future Research

Future research would ideally have a long-term follow-up on patients to determine whether OPL or OSCC develop. This would allow the ability to determine whether specific FISH patterns are associated with the development of OPL, dysplasia, OSCC, and possibly to the recurrence and aggressiveness of OSCC. The sensitivity and specificity of the cytobrush technique with FISH must also be evaluated. It has yet to be established whether these procedures are sensitive enough to detect clinically significant levels of genetic alterations, and also if it is specific enough to distinguish between genetically healthy individuals and genetically high-risk individuals for OSCC over a large and genetically diverse population.

7. APPENDICES

7.1 Ethics Approval Letter

SIMON FRASER UNIVERSITY

OFFICE OF RESEARCH ETHICS

BURNABY, BRITISH COLUMBIA
CANADA V5A 1S6
Telephone: (604) 291-3447
FAX: (604) 268-6785

August 30, 2005

Dr. Miriam Rosin
School of Kinesiology
Simon Fraser University

Dear Dr. Rosin

Re: Clonal changes in oral lesions of high-risk patients - Ref. #35159
National Institute of Dental & Craniofacial Research (NIH)
Amendment

In response to your request dated August 29, 2005, I am pleased to approve, on behalf of the Research Ethics Board, the minor amendment for the addition of collaborators, Ricky Chi-Wai Lee, Ling-I Tseng, Denise Laronde and L. Zhang in the research protocol of the above referenced Request for Ethical Approval of Research originally approved on May 19, 1998.

Best wishes for continued success in this research.

Sincerely,

Dr. Hal Weinberg, Director
Office of Research Ethics

c: Robert Jinze Li, Goubin Rubin Sun,
Vicki Flemming, Ricky Chi-Wai Lee,
Ling-I Tseng, Denise Laronde,
L. Zhang - Collaborators

jmy

7.2 Sample Calculation of Pack-years

Pack-years are a measure of total tobacco use based on the total number of years an individual has smoked multiplied with the average number of cigarettes consumed per day. A single pack-year is defined as the equivalent of smoking 1 pack of cigarettes (20 cigarettes per pack), per day for a year (365 days per year). Thus, a single pack-year equals = (20 cigarettes) x (365 days/year) = 7300 cigarettes.

The smoking data from the oral health questionnaire takes into account the total number of years the patient has smoked up to the time of sample collection and the estimated average number of cigarettes smoked per day. Marijuana use was not considered in the smoking data.

Example of Calculation

Patient's age at the start of regular cigarette use: 18 years
Patient's age at the time of sample collection: 40 years
Total number of years smoking: 22 years
Estimated average cigarette consumption per day: 5 cigarettes

Total Tobacco Consumption

$$\frac{(22 \text{ years}) \times (365 \text{ days/year}) \times (5 \text{ cigarettes/day})}{(7300 \text{ cigarettes/pack-year})} = 5.5 \text{ pack-years or } 40150 \text{ cigarettes}$$

7.3 Oral Health Questionnaire

COMMUNITY ORAL STUDY QUESTIONNAIRE

NAME: _____ ID#: _____
Date of Birth: _____(YYYY/MM/DD) Date: _____(YYYY/MM/DD)
Age: _____ Sex: _____ If First Nations Status: specify # _____

1. In addition to being Canadian or a landed immigrant, what is your ethnic or cultural heritage? Check one box only:
   - ☐ White
   - ☐ First Nations. STATUS: Yes ☐; No ☐
   - ☐ East or South-east Asian (eg. China, Japan, Indonesia, Philippines, Vietnam)
   - ☐ South Asian (eg. India Pakistan, Sri Lanka)
   - ☐ Black
   - ☐ Other (Please Specify)_____

2. What is the language that you are most fluent in? Check one box only:
   - ☐ English
   - ☐ Chinese (Mandarin/Cantonese)
   - ☐ French
   - ☐ Spanish
   - ☐ Other (Please Specify): _____

3. a) What is the highest grade (or year) of high school or elementary school that you have completed?
   Grade ___ Never attended school ____

   b) How many years of post-secondary school have you completed (college, university)?
   Years ___ None __

4. What is your current marital status? Check one box only:
   - ☐ Married or Living Common Law
   - ☐ Divorced
   - ☐ Separated
   - ☐ Widowed
   - ☐ Never married

5. Are you currently living at one of the following hotels? Check one box only:
   - ☐ The Portland Hotel
   - ☐ The Sunrise
   - ☐ The Washington
   - ☐ The Regal
   - ☐ The New Stanley Fountain
   - ☐ The Marpole
   - ☐ None of the above

6. Who do you live with? Check one box only:
   - ☐ Alone
   - ☐ With family
   - ☐ With others

7. Are you currently working? Check one box only:
   - ☐ Yes
   - ☐ No

8. What is your annual family income before taxes. (Family is a group of individuals related by blood, marriage including common-law, or adoption, who currently share a common dwelling unit). Check one box only:
   - ☐ less than $12,000
   - ☐ 12,001 – 15,000
   - ☐ 15,001 – 20,000
   - ☐ 20,001 – 25,000
   - ☐ 25,001 – 30,000
   - ☐ 30,001 – 35,000
   - ☐ 35,001-40,000
   - ☐ 40,001-45,000
   - ☐ 45,001-55,000
   - ☐ greater than $55,000
   - ☐ Don't know
   - ☐ Decline to answer

9. In the past 12 months, did you (or your family) receive income from any of the following assistance programs:
   - ☐ Child Tax Benefit
   - ☐ Guaranteed Income Supplement or Spouse's Allowance
   - ☐ Income Assistance or Welfare
   - ☐ Disability Insurance
   - ☐ Employment Insurance
   - ☐ None of the Above

10. Have you smoked 100 or more cigarettes in your life? Check one box only
   - ☐ Yes
   - ☐ No

11. Have you ever used the following: (Please check all that apply)
   - ☐ Betel nut
   - ☐ Chewing Tobacco
   - ☐ Marijuana
   - ☐ Crack/Cocaine
   - ☐ Crystal Meth
   - ☐ Heroin
   - ☐ None of the Above

12. Do you now drink or have you ever consumed alcoholic beverages more than once or twice a year?
   - ☐ Yes
   - ☐ No

Please specify who completed questionnaire:
Self ☐; Family Member ☐; Other ☐Please specify_____
OHN Staff ☐ (Name) _____

ADDENDUM SHEET TO DTES QUESTIONNAIRE

ID: _____ Name: _____ Date:_____ (YYYY/MM/DD)

1. TOBACCO:

	Did you ever smoke? (Y/N)	Do you currently smoke? (Y/N)	At what age did you begin to smoke?	If stopped at what age did you permanently stop?	Average Number per day? (Currently)	How many years in total did you smoke? (not including years where you didn't smoke)
Cigarettes** > 100 in lifetime						
Marijuana						

****Please specify if person reverse smokes**

If person ever used betel nut or chewing tobacco, please use "Chewers Addendum 20040123"

2. DRUG USE

	Did you ever use?	Do you currently use?	Method of use?	At what age did you begin to use?	If stopped at what age did you permanently stop	Average = per ___
Crack/Cocaine						
Crystal Meth						
Heroin						
Methadone						

3 ALCOHOL CONSUMPTION

	Did you ever consume?	Are you currently consuming?	At what age did you begin to consume?	If stopped at what age did you permanently stop?	Average Number per week?	How many years in total did you consume? (not including years where you didn't drink)
Beer (bottles)						
Wine (glasses)						
Spirits/Liquor (shots)						
Others (specify)						

4. FAMILY HISTORY

Have any of the patient's **immediate** family members (parents, brothers/sisters, daughters/sons, grandparents, aunts/uncles related by birth not marriage) had cancer in the head and neck region (excluding skin cancer)?

☐ Yes (Please indicate which relative and type of cancer) _____

☐ No

5. CURRENT LIVING ACCOMODATION – WHERE DOES PATIENT LIVE (ie/ homeless shelter, detox, on the street, apartment, senior complex, etc....)_____

☐ DTES (postal V6A or V6B) ☐ DTES – no fixed address ☐ Outside DTES

Please specify who completed addendum: OHN member name:_____

Tracking Sheet

Pat. ID= Name Date

		A	B
LESION	**CLINICAL LESION PRESENT:** 0 = No or 1= Yes		
	CLINICAL DESCRIPTION OF LESION- 1= OPL/Cancer; 2=inflammatory; 3= reactive/trauma; 4=others (ex;pigmentation); 5=NA		
	LESION SITE: R=right;L=left; U		
	LESION GRID LOCATION: Specify grid site.		
	LESION TYPE- ie. diffuse, discrete, scar only etc.		
	LENGTH (MM):		
	WIDTH (MM):		
	THICKNESS (MM):		
	COLOR: 0 = Normal; 1= White; 2=Predominantly (>50%) white; 3= Predominantly (>50%) red; 4 = Red; 5= Other, specify		
	APPEARANCE: 1 = Homogenous 2 = Nonhomogenous		
	TOLUIDINE BLUE. 0=Neg; 1=Pos; 2=Equiv; 3=Not done;		
	CLINICAL IMPRESSION, IF AVAILABLE		
GOGGLE	**GOGGLES- :** 0=Neg; 1=Pos; 2=Equiv; 3=Not done; 4=N/A (not applicable); 5= changed for other reason (confounders)		
	GOGGLE GRID LOCATION: (Specify where on grid)		
	GOGGLE LENGTH (MM):		
	GOGGLE WIDTH (MM):		
	PRESENCE OF ORANGE FLUORESCENCE: 1 = Yes 0 = No		
	GOGGLE DIGITAL IMAGE TAKEN: 1 = Yes 0 = No		
	GOGGLE COMMENT: 1=YES; 0=NO; 99= N/A (IF YES, PLEASE SEE ORAL MEDICINE SHEET)		
SAMPLE	**CHEEK CYTOBRUSH (BILATERAL BUCCAL MUCOSA)** 1=YES; 0=NO (If not from stated site, please specify, _____)		
	TONGUE CYTOBRUSH (BILATERAL LATERAL/VENTRAL TONGUE) 1=YES; 0=NO (If not from stated site, please specify,_____)		
	LESION BRUSH: 1=YES; 0=NO		
	WASH: 1=YES; 0=NO		
	PHOTO DONE (WHITE LIGHT): 1=YES; 0=NO		
	CONCURRENT BIOPSY 0=NO ; 1=YES, CONCURRENT; 2=YES, PREVIOUS, DX_____		
	IMMUNOCOMPROMISED: 1 = Yes 0 = No		
	SCRAPER- who did the scrape procedures?		
	CLINICAL COMMENT: 0=no ; 1=F/U ONLY;2=F/U WITH Tx; 3= REFER TO MD;4=Others;		

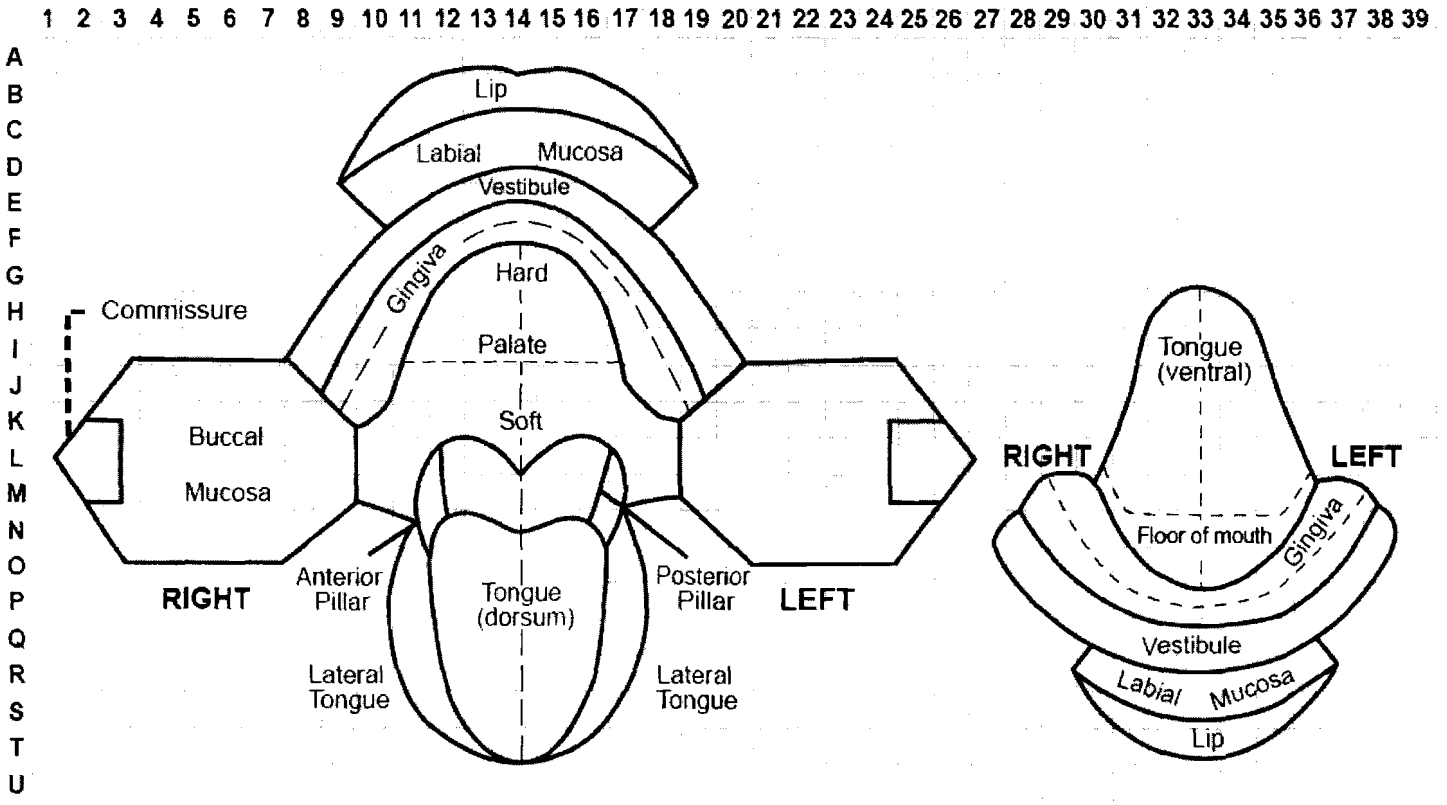
LOCATION OF LESION AND SAMPLES:

Patient Name: _____ _____ Date: _____
(Surname) *(First Name)* *(year/month/day)*

EXAMINER'S SIGNATURE _____ Date: _____

PRINCIPAL INVESTIGATOR'S SIGNATURE _____ Date: _____

EACH GRID BLOCK REPRESENTS 10mm x 10mm INDICATE LESION LOCATION



7.4 Incidence of Disease in Portland Community Clinic Patients

Path #	HepB*	HepC*	TB*	HIV*	Immuno-suppressed**
4081	0	0	0	0	0
4082	0	0	0	0	0
4083	0	0	0	0	0
4085	0	0	0	0	1
4086	0	0	1	0	0
4087	0	0	0	0	0
4089	0	1	0	1	2
4091	0	0	0	0	0
4092	0	1	0	0	1
4093	0	1	0	0	1
4094	0	0	0	0	0
4095	1	1	1	1	2
4096	0	0	0	0	0
4098	1	0	0	0	1
4099	0	0	0	0	0
4100	0	1	0	0	1
4102	0	0	0	0	0
4104	0	0	0	0	0
4105	0	0	0	0	0
4112	0	0	0	0	0
4115	0	0	1	0	0
4116	0	0	0	0	0
4117	0	0	0	0	0
4120	0	0	0	0	0
4123	0	0	0	0	0
4125	1	1	0	0	1
4126	0	0	0	0	0

Path #	HepB*	HepC*	TB*	HIV*	Immuno-suppressed**
4129	0	0	0	0	0
4131	0	0	1	0	0
4132	0	0	0	0	0
4133	0	0	1	0	0
4135	0	1	0	0	1
4136	0	1	0	0	1
4137	0	1	0	0	1
4138	0	0	0	0	0
4139	0	0	0	0	0
4140	1	1	0	1	2
4143	1	1	0	1	2
4144	0	0	0	0	0
4146	0	1	1	1	2
4147	0	0	1	0	0
4151	0	0	0	0	0
4152	0	0	0	0	0
4153	0	0	0	0	0
4154	0	0	0	0	0
4155	0	0	0	0	1
4157	0	1	0	0	1
4158	1	0	0	0	1
4159	0	0	0	0	0
4160	0	0	0	0	1
4161	0	0	0	0	1
4162	0	0	0	0	0
4165	1	1	0	0	1
Total	7	14	7	5	1=15, 2=5

* 0 = no, 1 = yes

** 0 = normal, 1 = medically complicated, 2 = immuno-suppressed and medically complicated. Assessed by Dr. Catherine Poh. "Medically complicated" determined by hepatitis infection or a declared disease or infection. Immunosuppression defined by HIV/AIDS infection.

7.5 Demographics, Smoking and Alcohol Data for ADC Patients

Patient #	Age (Years)	Gender	Smoking History[a]	Alcohol[b]	Pack-years
8166	60	M	S	Yes	50
8167	50	M	S	Yes	15.5
8168	59	F	NS	Yes	0
8169	49	F	S	Yes	37.75
8170	37	M	NS	Yes	0
8172	66	M	NS	Yes	0
8173	31	F	NS	Yes	0
8174	37	F	NS	Yes	0
8175	65	M	S	Yes	35.25
8176	43	M	S	Yes	11
8177	72	F	NS	Yes	0
8178	53	M	NS	No	0
8179	56	F	NS	Yes	0
8180	66	M	FS	Yes	14
8181	70	M	NS	Yes	0
8529	51	F	FS	Yes	7
8533	31	F	S	Yes	11.3
8538	37	F	NS	No	0
8540	55	F	NS	No	0
8542	80	F	FS	Yes	28
8545	54	M	NS	No	0
8548	47	F	NS	No	0
8551	50	F	NS	No	0
8553	55	F	NS	No	0
8555	48	M	S	Yes	33.5
8556	48	M	S	Yes	81
8557	58	F	FS	Yes	30.8
8558	43	M	NS	No	0
8559	33	F	NS	No	0
8562	53	M	FS	Yes	50
8563	59	M	NS	No	0
8567	31	F	S	Yes	50.6
8569	53	F	NS	No	0
8572	52	F	FS	Yes	1.5
8573	53	M	FS	Yes	22.5
8575	30	F	FS	Yes	3.3
8576	49	M	NS	No	0
8578	53	M	FS	Yes	60
8580	49	F	S	Yes	25
8584	47	M	S	Yes	40
8585	44	M	NS	No	0
8586	63	F	NS	Yes	25.4
8589	57	F	FS	Yes	25.5
8590	52	F	NS	No	0

Patient #	Age (Years)	Gender	Smoking History[a]	Alcohol[b]	Pack-years
8591	52	M	FS	Yes	15
8592	57	M	NS	No	0
8593	53	F	NS	No	0
8598	53	F	S	Yes	35.8
8599	58	F	NS	No	0
8600	62	F	NS	No	0
8601	48	M	NS	No	0

[a] S = current smoker, FS = former smoker of 6 months or longer, NS = non-smoker

[b] Alcohol consumption defined as exceeding 3 alcoholic beverages annually

7.6 Demographics, Smoking and Alcohol Data for PCC Patients

Patient #	Age (Years)	Gender	Smoking History[a]	Alcohol[b]	Pack-years
4081	36	M	S	No	19.5
4082	42	F	S	No	25
4083	35	M	NS	Yes	0
4085	52	M	FS	Yes	16.5
4086	54	M	S	Yes	24.75
4087	61	M	NS	No	0
4089	51	F	S	No	42
4091	57	M	S	Yes	47
4092	41	M	S	Yes	22
4093	44	F	S	Yes	6.5
4094	39	M	S	Yes	2
4095	44	M	S	Yes	13
4096	56	F	S	Yes	1.357
4098	40	F	S	Yes	12
4099	44	F	S	Yes	1.25
4100	43	F	S	Yes	24
4102	56	M	S	Yes	40
4104	73	F	NS	No	0
4105	47	M	S	Yes	21
4112	62	F	NS	No	0
4115	58	M	S	Yes	11
4116	58	M	S	No	30
4117	45	M	S	Yes	29
4120	62	M	NS	Yes	0
4123	53	M	S	Yes	8.05
4125	35	F	S	Yes	15
4126	44	M	S	Yes	2.2
4129	57	M	S	Yes	0.1
4131	51	F	NS	Yes	0
4132	47	F	S	Yes	8.25
4133	54	M	S	Yes	75.25
4135	49	M	S	Yes	7.6
4136	60	M	S	Yes	23
4137	52	M	S	Yes	3.5
4138	53	M	S	Yes	39
4139	53	M	S	Yes	20
4140	36	M	S	Yes	12.5
4143	45	M	S	Yes	37
4144	76	F	FS	Yes	26
4146	40	F	S	Yes	6.2
4147	54	M	S	Yes	13.65
4151	42	M	S	Yes	7.5
4152	49	M	S	Yes	5.1

Patient #	Age (Years)	Gender	Smoking History[a]	Alcohol[b]	Pack-years
4153	58	M	S	Yes	44
4154	61	M	S	Yes	47
4155	42	M	S	Yes	70
4157	57	M	S	Yes	10.4
4158	60	M	S	Yes	1.6
4159	45	M	S	Yes	8
4160	76	M	NS	No	0
4161	61	M	FS	Yes	92.5
4162	42	M	S	Yes	34
4165	54	M	S	Yes	39

[a] S = current smoker, FS = former smoker by more than 6 months, NS = non-smoker

[b] Alcohol consumption defined as exceeding 3 alcoholic beverages annually

7.7 Percentage of Cells with Specific FISH Signal Patterns for *3p14.2* in ADC Patients

Path #	Total Cells Scored	g2c2	g1c1	g2c1	g3c1	g4c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g3c4	g4c4
8166	435	92.0	0.7	0.9	0.5	0	0	3.2	1.1	0.5	0.5	0.7	0	0	0	0
8167	416	96.2	0.5	0.5	0	0	0	0.5	0.7	0.2	0.7	0.5	0.2	0	0	0
8168	409	97.8	0	0.7	0	0	0	0	0.7	0	0.2	0.5	0	0	0	0
8169	420	95.2	0.2	1.2	0	0	0	0.7	1.2	0.7	0	0.2	0	0	0.2	0.2
8170	416	96.2	0.2	0.2	0	0	0	1.4	1.7	0	0	0	0.2	0	0	0
8171	421	95.0	0	0.7	0.2	0	0	0.5	2.1	0.2	0.7	0.5	0	0	0	0
8172	415	96.4	0.2	1.0	0	0	0	1.0	1.0	0.2	0	0.2	0	0	0	0
8173	414	96.6	0.5	1.0	0	0	0	0.5	0.5	0.2	0.5	0.2	0	0	0	0
8174	423	94.6	0.2	1.4	0	0	0	0.9	0.9	0.5	0.5	0.7	0	0	0	0.2
8175	425	94.1	0.7	0.5	0.2	0	0	1.4	1.2	0.2	0.2	1.2	0.2	0	0	0
8176	421	95.0	0.2	1.2	0	0	0	1.7	1.0	0.2	0	0.5	0	0.2	0	0
8177	422	94.8	0.9	0.7	0	0	0	1.7	0.7	0.5	0	0.7	0	0	0	0
8178	420	95.2	0.2	1.0	0	0	0	1.0	1.2	0.7	0.7	0	0	0	0	0
8179	417	95.9	0	1.2	0	0	0	0.7	1.4	0.2	0.5	0	0	0	0	0
8180	416	96.2	0.2	0.5	0	0	0	1.0	0.7	0.2	1.0	0.2	0	0	0	0
8181	415	96.4	0.5	1.7	0	0	0	0	1.0	0	0.5	0	0	0	0	0
8533	509	94.3	0.4	0.6	0.2	0	0	2.2	1.0	0.6	0	0.4	0	0	0	0.4
8540	506	92.7	0.6	1.8	0	0	0	2.0	1.0	0.2	1.0	0.6	0	0	0	0.2
8541	508	96.3	0.2	1.4	0.2	0	0	0.6	0.4	0	0.6	0.4	0	0	0	0
8548	517	96.5	0	1.4	0	0	0	0.4	1.4	0.2	0.2	0	0	0	0	0
8549	387	94.1	0.3	0.8	0.3	0	0	0.8	1.3	1.6	0.8	0	0.3	0	0	0
8550	514	94.2	0.2	1.0	0	0	0	1.2	1.2	0.8	0.8	0.2	0.4	0.2	0	0
8553	471	95.3	0.2	1.7	0.2	0	0	1.1	0.8	0.6	0	0	0	0	0	0
8555	505	95.0	0.2	1.0	0	0	0	2.2	1.0	0.2	0.4	0	0	0	0	0
8556	500	94.2	0	1.8	0	0	0	1.0	1.2	0.4	0.2	1.2	0	0	0	0
8558	511	94.3	1.0	1.0	0	0	0	0.4	1.0	0.6	0.8	1.0	0	0	0	0
8559	500	94.8	0.2	1.4	0	0	0	1.2	0.8	0.4	0.6	0.4	0	0.2	0	0

Path #	Total Cells Scored	g2c2	g1c1	g2c1	g3c1	g4c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g3c4	g4c4
8560	500	96.0	0.4	0.8	0	0	0	1.0	1.0	0.2	0.4	0.2	0	0	0	0
8562	500	94.4	1.6	1.2	0.2	0	0	1.4	1.0	0	0	0.2	0	0	0	0
8567	496	95.0	0.2	0.8	0	0	0	1.0	2.0	0.6	0	0.4	0	0	0	0
8573	505	95.2	0.8	0.6	0	0	0	1.6	0.6	0.2	0.6	0.2	0	0	0	0.2
8575	521	95.0	0.6	1.3	0	0	0	1.2	1.0	0.4	0	0.4	0.2	0	0	0
8576	501	96.0	0.4	0.6	0	0	0	1.6	0.6	0	0.6	0.2	0	0	0	0
8578	484	95.5	0.2	1.2	0	0	0	0.4	1.9	0.4	0	0.4	0	0	0	0
8580	500	95.4	0	1.0	0.4	0	0	1.2	0.8	0.6	0.2	0.4	0	0	0	0
8584	499	97.0	0.2	0.6	0	0	0	0.4	0.8	0	0.6	0.2	0	0.2	0	0
8586	500	96.8	0.2	1.2	0	0	0	0.4	1.0	0.2	0	0.2	0	0	0	0
8590	496	94.4	0.2	1.6	0	0	0	0.8	1.4	0.6	0.2	0.6	0	0.2	0	0
8591	468	95.5	0	1.7	0	0	0.2	0.9	1.5	0	0.2	0	0	0	0	0
8592	407	93.4	0.5	1.2	0	0.2	0	2.0	1.2	0.2	0.5	0.2	0.2	0	0.2	0
8593	346	95.1	0.6	2.3	0	0	0	0.3	1.7	0	0	0	0	0	0	0
8596	500	95.6	0	1.2	0	0	0	0	1.8	0.6	0.2	0.6	0	0	0	0
8598	500	93.2	0.2	1.4	0.2	0	0.4	3.0	1.2	0.2	0.2	0	0	0	0	0
Average	462	95.2	0.3	1.1	0.1	0.0	0.0	1.1	1.1	0.3	0.3	0.3	0.0	0.0	0.0	0.0
# of patients with pattern	-	43	35	43	10	1	2	40	43	34	30	33	7	5	2	5
% of patients with patten	-	100	81.4	100	23.3	2.3	4.7	93.0	100	79.1	69.8	76.7	16.3	11.6	4.7	11.6

7.8 Percentage of Cells with Specific FISH Signal Patterns for *9p21* in ADC Patients

Path #	Total Cells Scored	g2c2	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g4c4
8166	431	92.8	0.2	0.9	0.9	0.2	2.8	0.2	0	0.7	0.5	0.2	0	0.5
8167	411	97.3	0	0.5	0.5	0.2	0.7	0	0.2	0	0.2	0	0	0.2
8168	403	99.3	0	0.2	0	0	0.5	0	0	0	0	0	0	0
8169	420	95.2	0	0.5	1.2	0	1.9	0	0.2	0.2	0	0.5	0	0.2
8170	406	98.5	0	0.5	0	0	0.7	0	0	0.2	0	0	0	0
8171	410	97.6	0	0.2	0	0	1.0	0.5	0	0.2	0.5	0	0	0
8172	404	99.0	0	0	0	0.2	0.5	0	0	0	0	0	0	0.2
8173	414	96.6	0	0	0	0.5	1.0	1.2	0	0	0	0.2	0	0.5
8174	404	99.0	0	0	0.2	0	0.2	0	0	0.5	0	0	0	0
8175	430	93.0	0	0.9	0.5	0.5	1.9	1.6	0.5	0.2	0	0	0	0.9
8176	418	95.7	0	0.5	0.7	0	1.7	0.5	0	0	0.2	0	0	0.7
8177	408	98.0	0	0	0.5	1.0	0.5	0	0	0	0	0	0	0
8178	410	97.6	0	0.2	0	0	0.7	0.2	0	0.5	0.2	0	0	0.5
8179	406	98.5	0.2	0	0	0	0.7	0.2	0.2	0	0	0	0	0
8180	415	96.4	0	0.2	0.5	0.7	1.7	0	0	0	0	0.2	0	0.2
8181	404	99.0	0	0.2	0.2	0	0.5	0	0	0	0	0	0	0
8529	410	97.6	0	0.5	0.2	0	1.5	0	0	0.2	0	0	0	0
8530	404	99.0	0	0	0	0	1.0	0	0	0	0	0	0	0
8533	412	97.1	0	1.2	0.2	0	1.0	0.5	0	0	0	0	0	0
8538	409	97.8	0	0.2	0.5	0	0.2	0.2	0	0.2	0.2	0.2	0	0.2
8540	442	95.5	0	0.5	0.9	0	2.0	0.2	0	0.2	0	0.2	0	0.5
8541	412	97.1	0	0.5	0.5	0.5	1.2	0	0	0	0	0	0	0.2
8545	412	97.1	0	0	0.5	0.2	1.5	0	0.2	0	0.2	0	0	0.2
8548	408	98.0	0	0	0	0.2	0.5	0	0	0.5	0	0	0	0.7
8550	411	97.3	0	0.2	0.2	0.2	1.2	0.2	0	0.2	0.2	0	0	0
8553	407	98.3	0.5	0.7	0	0.5	0	0	0	0	0	0	0	0
8555	418	97.4	0	0	1.0	0	1.2	0.2	0	0	0.2	0	0	0

Path #	Total Cells Scored	g2c2	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g4c4
8556	534	93.6	0.2	1.3	0.2	0.7	1.9	1.1	0.4	0	0.2	0.2	0	0.2
8558	411	97.3	0	1.0	0.7	0	0.5	0.5	0	0	0	0	0	0
8559	410	97.6	0	0.2	1.0	0	0.5	0.2	0	0	0.2	0	0	0.2
8560	408	98.0	0	0.5	0	0.5	0.7	0	0	0.2	0	0	0	0
8562	412	97.1	0	0.5	0	0	1.5	0.7	0	0	0	0	0	0.2
8563	414	98.3	0	0.5	0.2	0	1.0	0	0	0	0	0	0	0
8567	404	99.0	0	0.5	0	0	0.5	0	0	0	0	0	0	0
8569	408	98.0	0.2	0.0	0.2	0	1.2	0.2	0	0	0	0	0	0
8573	408	98.0	0	0.5	0.2	0.2	1.0	0	0	0	0	0	0	0
8575	411	93.7	0.2	0.7	0.5	0.5	3.4	0.7	0	0	0.2	0	0	0
8576	419	92.1	0	2.6	0.7	0.2	2.6	1.4	0	0	0.2	0	0	0
8578	409	97.8	0	0.7	0.2	0	1.2	0	0	0	0	0	0	0
8584	419	95.5	0	0.2	1.2	0.5	1.2	0.2	0	0.5	0	0	0.5	0.2
8585	406	98.5	0	0.5	0.2	0.2	0.5	0	0	0	0	0	0	0
8586	411	97.3	0	1.0	0.2	0.5	0.5	0.2	0	0.2	0	0	0	0
8592	406	98.5	0	0.0	0.2	0	0.7	0.2	0.2	0	0	0	0	0
8593	410	97.6	0	0.0	0.2	0	1.5	0	0.2	0	0	0	0	0.5
8596	405	98.8	0	0.5	0.2	0	0.5	0	0	0	0	0	0	0
8598	406	98.5	0	0.2	0.2	0	0.7	0	0	0	0.2	0	0	0
8599	345	98.3	0	0.3	0.3	0	0.9	0	0	0	0.3	0	0	0
8600	413	96.9	0.2	1.0	0.2	0	0.7	0	0.2	0	0.7	0	0	0
8601	405	98.8	0	0.2	0	0	1.0	0	0	0	0	0	0	0
Average	413	97.2	0	0.4	0.3	0.2	1.1	0.2	0.1	0.1	0.1	0	0	0.2
# of patients with pattern	-	49	7	37	35	20	48	22	9	15	16	7	1	19
% of patients with pattern	-	100	14.3	75.5	71.4	40.8	98.0	44.9	18.4	30.6	32.7	14.3	2.0	38.8

7.9 Percent of Cells with Chromosome and *3p14.2* Alterations in ADC Patients

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a] : Chromosome alterations only	Group B[b] : Gene alterations only	Group C[c] : All chromosome alterations	Group D[d] : All gene alterations	Group E[e] : Alterations in both gene and chromosome
8166	435	92.0	1.4	4.8	3.2	6.7	1.8
8167	416	96.2	1.2	1.4	2.4	2.6	1.2
8168	409	97.8	1.0	0.7	1.5	1.2	0.5
8169	420	95.2	1.2	2.6	2.1	3.6	1.0
8170	416	96.2	0.2	3.1	0.7	3.6	0.5
8171	421	95.0	1.4	2.9	2.1	3.6	0.7
8172	415	96.4	1.0	2.2	1.4	2.7	0.5
8173	414	96.6	1.4	1.2	2.2	1.9	0.7
8174	423	94.6	1.9	2.4	3.1	3.5	1.2
8175	425	94.1	0.7	2.8	3.1	5.2	2.4
8176	421	95.0	1.4	2.9	2.1	3.6	0.7
8177	422	94.8	0.7	2.8	2.4	4.5	1.7
8178	420	95.2	1.7	2.9	1.9	3.1	0.2
8179	417	95.9	1.7	2.4	1.7	2.4	0
8180	416	96.2	1.4	1.9	1.9	2.4	0.5
8181	415	96.4	2.2	1.0	2.7	1.4	0.5
8533	509	94.3	0.6	3.7	2.0	5.1	1.4
8540	506	92.7	2.8	3.2	4.2	4.5	1.4
8541	508	96.3	2.0	1.0	2.8	1.8	0.8
8548	517	96.5	1.5	1.9	1.5	1.9	0
8549	387	94.1	1.6	3.6	2.3	4.4	0.8
8550	514	94.2	1.9	3.1	2.7	3.9	0.8
8553	471	95.3	1.7	2.5	2.1	3.0	0.4
8555	505	95.0	1.4	3.4	1.6	3.6	0.2
8556	500	94.2	2.0	2.6	3.2	3.8	1.2
8558	511	94.3	1.8	2.0	3.7	3.9	2.0
8559	500	94.8	2.2	2.4	2.8	3.0	0.6
8560	500	96.0	1.2	2.2	1.8	2.8	0.6
8562	500	94.4	1.2	2.4	3.2	4.4	2.0
8567	496	95.0	0.8	3.6	1.4	4.2	0.6
8573	505	95.2	1.2	2.4	2.4	3.6	1.2
8575	521	95.0	1.3	2.5	2.5	3.6	1.2
8576	501	96.0	1.2	2.2	1.8	2.8	0.6
8578	484	95.5	1.2	2.7	1.9	3.3	0.6
8580	500	95.4	1.2	2.6	2.0	3.4	0.8
8584	499	97.0	1.4	1.2	1.8	1.6	0.4
8586	500	96.8	1.2	1.6	1.6	2.0	0.4
8590	496	94.4	2.0	2.8	2.8	3.6	0.8
8591	468	95.5	1.9	2.6	1.9	2.6	0
8592	407	93.4	1.7	3.4	3.2	4.9	1.5
8593	346	95.1	2.3	2.0	2.9	2.6	0.6
8596	500	95.6	1.4	2.4	2.0	3.0	0.6
Average	461	95.2	1.5	2.5	2.3	3.3	0.8

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g2c3, g3c3, g4c3, g2c4, g4c4

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g3c3, g4c3, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g3c3, g4c3, g4c4

7.10 Percent of Cells with Chromosome and *9p21* Alterations in ADC Patients

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a] : Chromosome alterations only	Group B[b] : Gene alterations only	Group C[c] : All chromosome alterations	Group D[d] : All gene alterations	Group E[e] : Alterations in both gene and chromosome
8166	431	92.8	1.6	3.2	3.9	7.0	2.3
8167	411	97.3	0.5	1.2	1.5	2.2	1.0
8168	403	99.3	0	0.5	0.2	0.7	0.2
8169	420	95.2	1.4	2.1	2.6	3.8	1.2
8170	406	98.5	0.2	0.7	0.7	1.7	0.5
8171	410	97.6	0.2	1.5	1.0	2.7	0.7
8172	404	99.0	0	0.7	0.2	1.0	0.2
8173	414	96.6	0	2.7	0.7	3.4	0.7
8174	404	99.0	0.7	0.2	0.7	1.2	0
8175	430	93.0	0.7	4.4	2.6	6.7	1.9
8176	418	95.7	0.7	2.2	2.2	3.6	1.4
8177	408	98.0	0.5	1.5	0.5	1.5	0
8178	410	97.6	0.5	1.0	1.5	2.9	1.0
8179	406	98.5	0	1.2	0.2	1.5	0.2
8180	415	96.4	0.5	2.4	1.2	3.1	0.7
8181	404	99.0	0.2	0.5	0.5	0.7	0.2
8529	410	97.6	0.5	1.5	1.0	2.4	0.5
8530	404	99.0	0	1.0	0	1.0	0
8533	412	97.1	0.2	1.5	1.5	2.7	1.2
8538	409	97.8	0.7	0.5	1.7	2.0	1.0
8540	442	95.5	1.1	2.3	2.3	3.8	1.1
8541	412	97.1	0.5	1.7	1.2	2.4	0.7
8545	412	97.1	0.5	1.9	1.0	2.4	0.5
8548	408	98.0	0.5	0.7	1.2	2.5	0.7
8550	411	97.3	0.5	1.7	1.0	2.7	0.5
8553	407	98.3	0	0.5	1.2	1.7	1.2
8555	418	97.4	1.0	1.4	1.2	1.7	0.2
8556	534	93.6	0.2	4.1	2.2	6.2	2.1
8558	411	97.3	0.7	1.0	1.7	1.9	1.0
8559	410	97.6	1.0	0.7	1.7	1.5	0.7
8560	408	98.0	0.2	1.2	0.7	2.2	0.5
8562	412	97.1	0	2.2	0.7	2.9	0.7
8563	414	98.3	0.2	1.0	0.7	1.4	0.5
8567	404	99.0	0	0.5	0.5	1.0	0.5
8569	408	98.0	0.2	1.5	0.5	1.7	0.2
8573	408	98.0	0.2	1.2	0.7	1.7	0.5
8575	411	93.7	0.5	4.6	1.7	5.8	1.2
8576	419	92.1	0.7	4.3	3.6	7.2	2.9
8578	409	97.8	0.2	1.2	1.0	2.0	0.7
8584	419	95.5	2.1	1.9	2.6	3.3	0.5

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a] : Chromosome alterations only	Group B[b] : Gene alterations only	Group C[c] : All chromosome alterations	Group D[d] : All gene alterations	Group E[e] : Alterations in both gene and chromosome
8585	406	98.5	0.2	0.7	0.7	1.2	0.5
8586	411	97.3	0.5	1.2	1.5	2.7	1.0
8592	406	98.5	0.2	1.2	0.2	1.2	0
8593	410	97.6	0.2	1.7	0.7	2.2	0.5
8596	405	98.8	0.2	0.5	0.7	1.0	0.5
8598	406	98.5	0.2	0.7	0.7	1.2	0.5
8599	345	98.3	0.3	0.9	0.9	1.4	0.6
8600	413	96.9	0.2	1.0	2.2	2.9	1.9
8601	405	98.8	0	1.0	0.2	1.2	0.2
Average	413	97.2	0.5	1.5	1.2	2.5	0.8

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4, g2c5

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g0c3, g1c3, g2c3, g3c3, g4c3, g1c4, g2c4, g3c4, g4c4, g2c5

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g0c3, g1c3, g3c3, g4c3, g1c4, g3c4, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g0c3, g1c3, g3c3, g4c3, g1c4, g3c4, g4c4

7.11 Percentage of Cells with Specific FISH Signal Pattern for *3p14.2* in PCC Patients

Path #	Total Cells Scored	g2c2	g1c0	g2c0	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g4c4
4081	467	85.7	0	1.1	0	1.1	7.7	0.2	0.9	2.8	0.2	0.2	0.2	0	0	0
4082	442	90.5	0	0.2	0	0.5	3.8	0	2.7	1.6	0.5	0.2	0	0	0	0
4083	429	93.2	0	0	0	1.6	3.5	0	0.5	1.2	0	0	0	0	0	0
4085	442	90.5	0.2	0.2	0	1.8	2.5	0	1.8	2.9	0	0	0	0	0	0
4086	480	83.3	1.9	1.7	0.21	2.7	5.2	0	1.3	2.7	0.6	0	0.4	0	0	0
4089	437	91.5	0	0.2	0	0	1.6	0	2.3	4.1	0.2	0	0	0	0	0
4091	458	87.3	0	1.1	0	0	3.3	0	0.7	7.0	0	0.2	0.4	0	0	0
4092	448	89.3	0	0.4	0	1.1	1.8	0	1.1	5.6	0	0.2	0.2	0.2	0	0
4093	422	94.8	0	0	0	0	3.3	0	0.7	0.7	0.2	0	0	0	0	0.2
4094	436	91.7	0	0.5	0	1.8	4.4	0	0.7	0.9	0	0	0	0	0	0
4095	444	90.1	0	0	0	0.9	2.5	0	1.1	4.7	0.5	0	0	0	0	0.2
4096	456	87.7	0.2	1.5	0	0.2	2.9	0	2.2	3.9	0	0.2	1.1	0	0	0
4098	442	90.5	0	0.5	0	1.8	3.8	0	2.3	0	1.1	0	0	0	0	0
4099	487	82.1	0.2	1.0	0	2.5	7.6	0	2.5	3.1	0.2	0.2	0	0	0	0.6
4100	413	96.9	0	0.2	0	0	1.2	0	1.0	0.2	0.2	0	0	0	0	0.2
4102	460	87.0	0.2	0	0	5.0	4.8	0	2.6	0.2	0	0	0	0	0.2	0
4104	420	95.2	0	0.2	0	0.7	1.0	0	1.0	1.9	0	0	0	0	0	0
4105	445	89.9	0	0.7	0	1.3	4.3	0.2	2.5	1.1	0	0	0	0	0	0
4112	416	96.2	0	0	0	0.5	0.2	0	0.7	1.0	0	1.4	0	0	0	0
4115	434	92.2	0	0	0	1.4	2.5	0	2.3	0.9	0.7	0	0	0	0	0
4116	444	90.1	0.2	0.7	0	2.7	2.7	0	3.4	0.2	0	0	0	0	0	0
4117	443	90.3	0	0.5	0	2.7	3.8	0	1.4	0.2	0	0.2	0.9	0	0	0
4120	444	90.1	0	0	0	5.6	1.1	0	2.9	0	0.2	0	0	0	0	0
4123	445	89.9	0	4.9	0	0.7	2.2	0	0	1.1	0	0	0.9	0	0	0.2
4125	414	96.6	0	0	0	1.0	0.2	0	0.5	1.7	0	0	0	0	0	0
4126	410	97.6	0	0	0	0.7	0.2	0	1.2	0	0	0	0	0	0	0.2
4129	413	96.9	0	0.2	0	1.0	0.7	0	1.0	0.2	0	0	0	0	0	0

Path #	Total Cells Scored	g2c2	g1c0	g2c0	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g2c3	g3c3	g4c3	g2c4	g4c4
4131	428	93.5	0	0	0	1.6	0.2	0	1.2	2.8	0	0	0.2	0	0	0.5
4132	439	91.1	0	0	0	1.1	4.8	0	0.7	0.5	0.5	1.1	0	0	0	0.2
4133	433	92.4	0	0	0	1.2	3.5	0	2.3	0	0	0	0.5	0	0	0.2
4136	437	91.5	0	0	0	2.7	1.6	0	2.5	1.1	0	0	0.5	0	0	0
4137	431	92.8	0.2	0.2	0	1.6	1.4	0	2.8	0	0	0	0.9	0	0	0
4138	450	88.9	0	0	0	1.3	3.8	0	1.8	2.9	0	1.3	0	0	0	0
4139	448	89.3	0.2	2.5	0	0.9	5.1	0	1.1	0.9	0	0	0	0	0	0
4140	436	91.7	0	0.2	0	1.8	3.4	0	0.7	1.6	0	0.5	0	0	0	0
4143	440	90.9	0.5	0.9	0	3.0	2.7	0.2	0.7	1.1	0	0	0	0	0	0
4144	451	88.7	1.1	2.0	0	0.9	4.7	0	1.1	0.4	0	0	1.1	0	0	0
4146	432	92.6	0	0.5	0	1.2	3.7	0	1.6	0.2	0	0	0.2	0	0	0
4147	452	88.5	1.5	3.3	0	2.7	3.8	0	0	0.2	0	0	0	0	0	0
4151	438	91.3	0	1.1	0	1.4	4.8	0	0.2	0	0	0.2	0.9	0	0	0
4152	433	92.4	0.2	1.2	0	0.7	5.3	0	0.2	0	0	0	0	0	0	0
4153	447	89.5	0.9	1.3	0	2.9	2.7	0	1.6	1.1	0	0	0	0	0	0
4154	421	95.0	0	0	0	3.1	1.2	0	0.5	0	0	0	0.2	0	0	0
4155	480	83.3	3.8	5.2	0	2.5	1.5	0.4	2.3	0	0	0	1.0	0	0	0
4157	464	86.2	0	9.3	0	0.6	3.0	0	0.9	0	0	0	0	0	0	0
4158	434	92.2	0.9	1.2	0	0.5	3.0	0	0.2	0.9	0	0	0.9	0	0	0.2
4159	412	97.1	0.2	0	0	0.7	1.5	0	0	0.5	0	0	0	0	0	0
4160	427	93.7	0.5	0.9	0	1.6	0.7	0	0.9	1.2	0	0	0.5	0	0	0
4161	421	95.0	0.2	0.5	0	0.7	1.0	0	0.7	1.4	0	0	0.5	0	0	0
4162	421	95.0	0	0.7	0	1.0	1.4	0	0.5	0.7	0.7	0	0	0	0	0
Average	439	91.2	0.3	0.9	0.0	1.5	2.9	0.0	1.3	1.4	0.1	0.1	0.2	0.0	0.0	0.1
# of patients with pattern	-	49	18	34	1	46	50	4	47	40	13	12	19	1	1	10
% of patients with pattern	-	100	36	68	2	92	100	8	94	80	26	24	38	2	2	20

7.12 Percentage of Cells with Specific FISH Signal Pattern for *9p21* in PCC Patients

Path #	Total Cells Scored	g2c2	g1c0	g2c0	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g0c3	g1c3	g2c3	g3c3	g4c3	g1c4	g2c4	g3c4	g4c4	g2c5
4081	426	93.9	0	0	0	0.9	0.9	0.7	2.8	0.2	0	0	0	0	0.2	0	0	0	0	0.2	0
4082	425	94.1	0	0	0	1.2	0.2	1.9	1.6	0.9	0	0	0	0	0	0	0	0	0	0	0
4083	434	92.2	0	0	0	0	0	0.9	5.8	0	0	0	0	1.2	0	0	0	0	0	0	0
4085	495	80.8	0	1.2	0	1.8	1.6	2.6	4.6	1.8	0.4	0	0	2.8	1.0	0	0	1.2	0	0	0
4086	431	92.8	0	0	0.2	0.9	0.5	1.4	3.0	0.2	0	0	0	0.2	0.7	0	0	0	0	0	0
4087	408	98.0	0	0	0	1.0	0	0	0	0.5	0	0	0	0	0.2	0	0	0	0	0.2	0
4089	428	93.5	0	0	0	0.9	0.2	1.6	3.0	0	0	0	0	0.5	0	0	0	0.2	0	0	0
4091	433	92.4	0	0	0	1.6	0	1.2	3.5	0.2	0	0	0	0.5	0	0	0.2	0	0.2	0.2	0
4092	440	90.9	0.2	0	0	0.7	0.5	1.4	5.9	0.2	0	0	0	0	0	0	0	0.2	0	0	0
4093	439	91.1	0	0	0	0	0.5	2.3	4.6	0.7	0	0	0	0	0.2	0	0	0.2	0	0.5	0
4094	426	93.9	0	0	0	0.2	0	3.3	2.1	0	0	0	0	0.2	0	0	0	0	0	0.2	0
4095	421	95.0	0	0	0	0	0	1.0	2.1	0.2	0	0	0	1.0	0.5	0	0	0	0	0.2	0
4096	450	88.9	0	0	0	0.2	0.9	6.7	2.0	0.2	0	0	0	0.9	0	0	0	0	0	0.2	0
4098	427	93.7	0	0	0	0.5	0	3.3	1.6	0	0	0	0	0.5	0.5	0	0	0	0	0	0
4099	428	93.5	0	0.2	0	0.7	0.2	2.3	2.3	0.5	0	0	0	0.2	0	0	0	0	0	0	0
4100	426	93.9	0	0.5	0	0.2	0	2.6	1.6	0.2	0	0	0	0.7	0.2	0	0	0	0	0	0
4102	450	88.9	0	0	0	0.4	0.7	1.3	2.4	0.2	0	0	0.4	2.2	0.2	0	0	2.9	0	0.2	0
4104	452	88.5	0	0	0	0.2	0	3.8	4.2	2.0	0	0	0	1.3	0	0	0	0	0	0	0
4105	441	90.7	0	0	0	0	0	2.0	4.1	0.7	0	0.2	0.5	1.4	0	0	0	0.5	0	0	0
4112	452	88.5	0	0	0	0	0.2	2.0	2.7	0.7	0	0.2	0.7	2.0	0	0	0	2.7	0.2	0.2	0
4115	428	93.5	0	0	0	0	0	1.9	4.7	0	0	0	0	0	0	0	0	0	0	0	0
4116	424	94.3	0	0	0.2	0	0	0.7	4.0	0	0	0	0	0.7	0	0	0	0	0	0	0
4117	452	88.5	0	0	0	0	0.2	7.7	3.3	0	0	0	0	0	0.2	0	0	0	0	0	0
4120	417	95.9	0	0	0	0	0.5	1.7	1.7	0	0	0	0	0	0	0	0	0	0	0	0.2
4123	432	92.6	0	0	0	0	0	3.0	3.9	0	0	0	0	0.2	0.2	0	0	0	0	0	0
4125	488	82.0	0	0	0.6	0.4	0	9.0	6.8	0.2	0	0.6	0.2	0.2	0	0	0	0	0	0	0

Path #	Total Cells Scored	g2c2	g1c0	g2c0	g0c1	g1c1	g2c1	g0c2	g1c2	g3c2	g4c2	g0c3	g1c3	g2c3	g3c3	g4c3	g1c4	g2c4	g3c4	g4c4	g2c5
4126	417	95.9	0	0	0	0	0	0.5	2.4	0.2	0.2	0	0	0.7	0	0	0	0	0	0	0
4129	445	89.9	0	0	0	0.9	0.2	4.7	3.8	0.2	0	0	0	0	0.2	0	0	0	0	0	0
4131	467	85.7	0	0	0	0.2	0	4.3	8.4	0.2	0	0	0.2	0.6	0.2	0	0	0	0	0.2	0
4132	448	89.3	0	0	0	0.7	0	0.7	7.4	0.2	0	0	0	0.9	0.7	0	0	0.2	0	0	0
4133	442	90.5	0	0	0	0	0	1.8	4.8	0	0	0	0.2	0.9	0.2	0	0.2	0.7	0	0.7	0
4135	451	88.7	0	0	0	0.9	0	4.4	2.9	0.9	0	0	0.2	1.1	0.2	0	0	0.2	0.2	0.2	0
4136	416	96.2	0	0	0	0	0.5	1.7	0.7	0.7	0	0	0	0.2	0	0	0	0	0	0	0
4137	455	87.9	0	0	0	4.4	0.2	2.0	3.5	1.3	0	0	0	0	0	0.2	0	0.2	0	0.2	0
4138	443	90.3	0	0	0.2	1.1	0.9	1.1	3.6	0.9	0.7	0	0	0.9	0.2	0	0	0	0	0	0
4139	418	95.7	0	0	0	0.2	0	0.7	1.9	0.2	0	0	0	1.0	0	0	0	0	0	0.2	0
4140	516	77.5	0	0	0	0.4	0	9.1	3.5	0	0	1.0	0.4	7.9	0	0	0	0	0.2	0	0
4143	420	95.2	0	0	0	0.7	0	0.2	2.6	0.2	0.2	0	0	0.7	0	0	0	0	0	0	0
4144	437	91.5	0	0.7	0	0.2	0.2	3.2	2.1	0	0	0	0.2	0	0.7	0	0	0.9	0	0.2	0
4146	427	93.7	0	0	0	0.9	0	0.7	2.1	0.9	0	0	0	0.7	0.7	0	0	0	0	0.2	0
4147	420	95.2	0	0	0	0.7	0	1.7	1.9	0.2	0	0	0	0.2	0	0	0	0	0	0	0
4151	434	92.2	0	0	0	2.8	0	0	1.2	0	0	0	0	3.9	0	0	0	0	0	0	0
4152	456	87.7	0	0	0	1.1	0	0.9	1.5	0.2	0	0	0.2	4.8	0	0	0	3.5	0	0	0
4153	447	89.5	0	0	0	0.9	0	5.6	3.6	0	0	0	0	0.2	0.2	0	0	0	0	0	0
4154	494	81.0	0	0	2.4	8.3	0.2	3.6	2.0	0.6	0	0	0	1.4	0	0	0	0	0	0.4	0
4155	486	82.3	0.2	0	0	0	0	10.7	2.9	1.2	0	0.2	0	2.1	0	0	0.2	0.2	0	0	0
4157	431	92.8	0	0	0	1.2	0	1.2	3.0	0	0	0.2	0.5	0.9	0	0	0	0.2	0	0	0
4158	438	91.3	0.2	0	0	0.2	0	1.6	3.0	0.7	0	0	0	2.1	0.9	0	0	0	0	0	0
4160	411	97.3	0	0	0	0	0	0.7	1.7	0.2	0	0	0	0	0	0	0	0	0	0	0
4161	478	83.7	0	0	0	5.2	0.6	0.6	5.6	0	0	0	0.4	3.3	0.4	0	0	0	0	0	0
4162	467	85.7	0.2	0	0	0	0	4.1	4.3	0	0	0	0	2.8	1.1	0	0	1.7	0	0.2	0
4165	480	83.3	0	0	0	4.0	0.8	0.6	5.4	0.6	0	0	0.8	4.0	0.2	0.2	0	0	0	0	0
Average	443	90.6	0.0	0.1	0.1	0.9	0.2	2.6	3.3	0.4	0.0	0.0	0.1	1.1	0.2	0.0	0.0	0.3	0.0	0.1	0.0
# of patients with pattern	-	51	4	4	5	36	21	50	51	35	4	6	13	40	24	2	3	16	4	18	1
% of patients with pattern	-	100	8	8	10	69	40	96	98	67	8	12	25	77	46	4	6	31	8	35	2

7.13 Percent of Cells with Chromosome and *3p14.2* Alterations in PCC Patients

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a]: Chromosome alterations only	Group B[b]: Gene alterations only	Group C[c]: All chromosome alterations	Group D[d]: All gene alterations	Group E[e]: Alterations in both gene and chromosome
4081	467	85.7	9.0	4.1	10.3	5.4	1.3
4082	442	90.5	4.3	4.8	4.8	5.2	0.5
4083	429	93.2	3.5	1.6	5.1	3.3	1.6
4085	442	90.5	2.7	4.8	4.8	6.8	2.0
4086	480	83.3	6.9	4.6	12.1	9.8	5.2
4089	437	91.5	1.8	6.6	1.8	6.6	0
4091	458	87.3	4.6	7.6	5.0	8.1	0.4
4092	448	89.3	2.5	6.7	4.0	8.3	1.6
4093	422	94.8	3.3	1.7	3.6	1.9	0.2
4094	436	91.7	4.8	1.6	6.7	3.4	1.8
4095	444	90.1	2.5	6.3	3.6	7.4	1.1
4096	456	87.7	4.6	6.1	6.1	7.7	1.5
4098	442	90.5	4.3	3.4	6.1	5.2	1.8
4099	487	82.1	8.8	5.7	12.1	9.0	3.3
4100	413	96.9	1.5	1.5	1.7	1.7	0.2
4102	460	87.0	5.0	2.8	10.2	8.0	5.2
4104	420	95.2	1.2	2.9	1.9	3.6	0.7
4105	445	89.9	4.9	3.8	6.3	5.2	1.3
4112	416	96.2	1.7	1.7	2.2	2.2	0.5
4115	434	92.2	2.5	3.9	3.9	5.3	1.4
4116	444	90.1	3.4	3.6	6.3	6.5	2.9
4117	443	90.3	4.5	1.6	8.1	5.2	3.6
4120	444	90.1	1.1	3.2	6.8	8.8	5.6
4123	445	89.9	7.2	1.1	9.0	2.9	1.8
4125	414	96.6	0.2	2.2	1.2	3.1	1.0
4126	410	97.6	0.2	1.2	1.2	2.2	1.0
4129	413	96.9	1.0	1.2	1.9	2.2	1.0
4131	428	93.5	0.2	4.0	2.6	6.3	2.3
4132	439	91.1	5.9	1.6	7.3	3.0	1.4
4133	433	92.4	3.5	2.3	5.3	4.2	1.8
4136	437	91.5	1.6	3.7	4.8	6.9	3.2
4137	431	92.8	1.6	2.8	4.4	5.6	2.8
4138	450	88.9	5.1	4.7	6.4	6.0	1.3
4139	448	89.3	7.6	2.0	8.7	3.1	1.1
4140	436	91.7	4.1	2.3	6.0	4.1	1.8
4143	440	90.9	3.6	2.0	7.0	5.5	3.4
4144	451	88.7	6.7	1.6	9.8	4.7	3.1
4146	432	92.6	4.2	1.9	5.6	3.2	1.4
4147	452	88.5	7.1	0.2	11.3	4.4	4.2
4151	438	91.3	6.2	0.2	8.4	2.5	2.3

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a]: Chromosome alterations only	Group B[b]: Gene alterations only	Group C[c]: All chromosome alterations	Group D[d]: All gene alterations	Group E[e]: Alterations in both gene and chromosome
4152	433	92.4	6.5	0.2	7.4	1.2	0.9
4153	447	89.5	4.0	2.7	7.8	6.5	3.8
4154	421	95.0	1.2	0.5	4.5	3.8	3.3
4155	480	83.3	6.7	2.7	14.0	10.0	7.3
4157	464	86.2	12.3	0.9	12.9	1.5	0.6
4158	434	92.2	4.1	1.2	6.7	3.7	2.5
4159	412	97.1	1.5	0.5	2.4	1.5	1.0
4160	427	93.7	1.6	2.1	4.2	4.7	2.6
4161	421	95.0	1.4	2.1	2.9	3.6	1.4
4162	421	95.0	2.1	1.9	3.1	2.9	1.0
Average	439	91.2	3.9	2.8	6.0	4.9	2.1

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g2c3, g3c3, g4c3, g2c4, g4c4

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g3c3, g4c3, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g3c3, g4c3, g4c4

7.14 Percent of Cells with Chromosome and *9p21* Alterations in PCC Patients

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a]: Chromosome alterations only	Group B[b]: Gene alterations only	Group C[c]: All chromosome alterations	Group D[d]: All gene alterations	Group E[e]: Alterations in both gene and chromosome
4081	426	93.9	0.9	3.8	2.3	5.2	1.4
4082	425	94.1	0.2	4.5	1.4	5.6	1.2
4083	434	92.2	1.2	6.7	1.2	6.7	0
4085	495	80.8	6.9	9.5	9.7	12.3	2.8
4086	431	92.8	0.7	4.6	2.6	6.5	1.9
4087	408	98.0	0	0.5	1.5	2.0	1.5
4089	428	93.5	0.9	4.7	1.9	5.6	0.9
4091	433	92.4	0.5	4.8	2.8	7.2	2.3
4092	440	90.9	0.7	7.5	1.6	8.4	0.9
4093	439	91.1	0.7	7.5	1.4	8.2	0.7
4094	426	93.9	0.2	5.4	0.7	5.9	0.5
4095	421	95.0	1.0	3.3	1.7	4.0	0.7
4096	450	88.9	1.8	8.9	2.2	9.3	0.4
4098	427	93.7	0.5	4.9	1.4	5.9	0.9
4099	428	93.5	0.7	5.1	1.4	5.8	0.7
4100	426	93.9	1.2	4.5	1.6	4.9	0.5
4102	450	88.9	5.8	4.0	7.1	5.3	1.3
4104	452	88.5	1.3	10.0	1.5	10.2	0.2
4105	441	90.7	1.8	6.8	2.5	7.5	0.7
4112	452	88.5	4.9	5.3	6.2	6.6	1.3
4115	428	93.5	0	6.5	0	6.5	0
4116	424	94.3	0.7	4.7	0.9	5.0	0.2
4117	452	88.5	0.2	11.1	0.4	11.3	0.2
4120	417	95.9	0.7	3.4	0.7	3.4	0
4123	432	92.6	0.2	6.9	0.5	7.2	0.2
4125	488	82.0	0.2	16.0	2.0	17.8	1.8
4126	417	95.9	0.7	3.4	0.7	3.4	0
4129	445	89.9	0.2	8.8	1.3	9.9	1.1
4131	467	85.7	0.6	12.8	1.5	13.7	0.9
4132	448	89.3	1.1	8.3	2.5	9.6	1.3
4133	442	90.5	1.6	6.6	2.9	7.9	1.4
4135	451	88.7	1.3	8.2	3.1	10.0	1.8
4136	416	96.2	0.7	3.1	0.7	3.1	0
4137	455	87.9	0.4	6.8	5.3	11.6	4.8
4138	443	90.3	1.8	6.3	3.4	7.9	1.6
4139	418	95.7	1.0	2.9	1.4	3.3	0.5
4140	516	77.5	7.9	12.6	9.9	14.5	1.9
4143	420	95.2	0.7	3.3	1.4	4.0	0.7
4144	437	91.5	1.8	5.3	3.2	6.6	1.4
4146	427	93.7	0.7	3.7	2.6	5.6	1.9

Path #	Total Cells Scored	% Cells with normal pattern	Group A[a]: Chromosome alterations only	Group B[b]: Gene alterations only	Group C[c]: All chromosome alterations	Group D[d]: All gene alterations	Group E[e]: Alterations in both gene and chromosome
4147	420	95.2	0.2	3.8	1.0	4.5	0.7
4151	434	92.2	3.9	1.2	6.7	3.9	2.8
4152	456	87.7	8.3	2.6	9.6	3.9	1.3
4153	447	89.5	0.2	9.2	1.3	10.3	1.1
4154	494	81.0	1.6	6.3	12.8	17.4	11.1
4155	486	82.3	2.3	14.8	2.9	15.4	0.6
4157	431	92.8	1.2	4.2	3.0	6.0	1.9
4158	438	91.3	2.1	5.3	3.4	6.6	1.4
4160	411	97.3	0	2.7	0	2.7	0
4161	478	83.7	4.0	6.3	10.0	12.3	6.1
4162	467	85.7	4.5	8.4	6.0	9.9	1.5
4165	480	83.3	4.8	6.7	10.0	11.9	5.2
Average	443	90.6	1.7	6.2	3.2	7.7	1.5

[a] Includes the following FISH signal patterns: g2c0, g2c1, g2c3, g2c4, g2c5

[b] Signal patterns: g0c2, g1c2, g3c2, g4c2

[c] Signal patterns: g1c0, g2c0, g0c1, g1c1, g2c1, g0c3, g1c3, g2c3, g3c3, g4c3, g1c4, g2c4, g3c4, g4c4, g2c5

[d] Signal patterns: g1c0, g0c1, g1c1, g0c2, g1c2, g3c2, g4c2, g0c3, g1c3, g3c3, g4c3, g1c4, g3c4, g4c4

[e] Signal patterns: g1c0, g0c1, g1c1, g0c3, g1c3, g3c3, g4c3, g1c4, g3c4, g4c4

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