MICROSATELLITE INSTABILITY AND LOSS OF HETEROZYGOSITY IN ORAL SQUAMOUS CELL CARCINOMA IN A MALAYSIAN POPULATION

by

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LIST OF ABBREVIATIONS

µl	Microlitre
A260/A280	Ratio of 260 absorbance over 280 absorbance
Bp	Base pair
Buffer AL	Lysis buffer AL
Buffer ATE	Elution buffer
Buffer ATL	Tissue lysis buffer
Buffer AW1	Wash buffer 1
Buffer AW2	Wash buffer 2
Buffer EB	Elution buffer
Buffer PB	Binding buffer
Buffer PE	Wash buffer
CDKN2A	Cyclin-dependent kinase inhibitor 2A
ddH_2O	Deionized distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor
FFPE	Formalin fixed paraffin embedded
FHIT	Fragile Histidine Triad
H&E	Hematoxylin and Eosin

HNPCC	Hereditary non-polyposis colon cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
LOH	Loss of heterozygosity
MA	Microsatellite alteration
Mb	Megabase
MD	Moderate differentiated
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
MLH1	MutL homolog 1
mM	Millimolar
MMR	Miss match repair
MSH2	MutS homolog 2
MSH6	MutS holomolog 6
MSI	Microsatellite Instability
ng/ml	Nanogram per microlitre
^{o}C	Degree Celsius
OSCC	Oral squamous cell carcinoma
p53	Protein 53
PCR	Polymerase chain reaction
PD	Poorly differentiated
PMS2	Post meiotic segregation increased 2

RFLP	Restrict fragment length polymorphism
SSCP	Single strand confirmation polymorphism
SSLP	Short tandem repeat polymorphism
STR	Simple tandem repeat
Taq	*Thermophilus aquaticus*
TBE	Tris/Borate/EDTA
TSG	Tumour suppressor gene
VHL	Von Hippel Lindau
WD	Well differentiated

KETIDAKSTABILAN MIKROSATELIT DAN KEHILANGAN HETEROZIGOSITI PADA KARSINOMA SEL SKUAMOSA MULUT DALAM POPULASI MALAYSIA

ABSTRAK

Kehilangan heterozigositi (KH) dan ketidakstabilan mikrosatelit (KM) telah didokumentasikan sebagai peristiwa penting dalam perkembangan karsinoma sel skuamosa mulut. Lima penanda mikrosatelit iaitu D3S192, D3S966, D3S647, D3S1228 dan D3S659 dipilih pada kromosom 3p kerana perubahan berfrekuensi tinggi yang dilaporkan dalam karsinoma sel skuamosa kepala dan leher dan penglibatan gen *VHL* (von Hippel Lindau) pada 3p25-26 dan gen *FHIT* (fragile histidine triad) pada 3p14.2 terbukti dalam pelbagai jenis tumor. Sebanyak 50 sampel tisu arkib karsinoma sel skuamosa mulut dan tisu normal pada pesakit yang sama diambil untuk analisis status KH dan KM. Pada keseluruhannya, KH yang terdapat pada kromosom 3p didapati dalam 56 daripada 189 kes (29.6%). KH yang paling sering didapati adalah pada penanda D3S966 dengan 18/42 (42.8%) kes menyarankan kehadiran TSGs pada lokasi ini. Dalam kajian ini, didapati KM pada D3S966 paling sering terjadi iaitu 28.6% kes, hal ini menunjukkan kemungkinan terjadinya mutasi pada gen MMR pada lokasi ini. Perubahan mikrosatelit paling sering terjadi pada D3S966 (71.4%), D3S1228 (56.7%) dan D3S192 (41.0%). Dalam kajian ini, tiada hubungan yang signifikan didapati antara KH pada kromosom 3p dengan jantina, peringkat tumor dan gred perbezaan tisu. Namun begitu, hubungan yang signifikan didapati diantara peringkat tumor dan perbezaan tisu dengan status KM dengan nilai *p* 0.002 dan 0.035. Terdapat juga hubungan yang

signifikan diantara perubahan mikrosatelit dengan gred berbezaan tisu dengan nilai *p* 0.041.

MICROSATELLITE INSTABILITY AND LOSS OF HETEROZYGOSITY IN ORAL SQUAMOUS CELL CARCINOMA IN A MALAYSIAN POPULATION

ABSTRACT

Loss of heterozygosity (LOH) and microsatellite instability (MSI) have been documented as important events of oral squamous cell carcinoma (OSCC). Five microsatellite markers D3S192, D3S966, D3S647, D3S1228 and D3S659 were selected on chromosome 3p because of high frequency of alterations reported in head and neck squamous cell carcinoma and the involvement of *VHL* (von Hippel Lindau) at 3p25-26 and the *FHIT* (fragile histidine triad) at 3p14.2 genes proven in many tumor types. A total of 50 archival tissue samples of OSCC and corresponding normal samples were analyzed for LOH and MSI status. The overall LOH for the markers selected on 3p was 56 out of 189 informative cases (29.6%). The most frequent LOH was identified at the marker D3S966 which is 18/42 (42.8%) of informative cases suggesting the presence of putative of TSGs in this loci. In this study, we found high MSI in D3S966 which is 28.6% of informative cases; this reveals the possibility of mutation of mismatch repair genes in this region. Frequent microsatellite alteration (MA) was observed in D3S966 (71.4%), D3S1228 (56.7%) and D3S192 (41.0%). There is no significant association between LOH with gender, tumour stage and differentiation grades. However, there is a significant association between tumour stage and differentiation grades with MSI status in OSCC in Malaysian population with *p* values of 0.002 and 0.035. There is also a significant association between MA and differentiation grades with a *p* value of 0.041.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Oral cancer remains a serious and constant problem. It is the sixth most common cancer reported worldwide with estimated 405,318 new cases annually. Markedly, higher prevalence of oral cancer is found in some Asian populations, mainly in South Asian countries including India and Pakistan, where it is the most common cancer in males and the second most common cancer in both sexes. Geographic or regional variations in the prevalence of oral cancer indicate that the socio-cultural lifestyles of a population play an important role in oral carcinogenesis. Squamous cell carcinoma accounts for more than 90% of oral cancers (Iamaroon et al., 2004).

In Peninsular Malaysia, Indian was the predominant ethnic group who had oral cancer in Kelantan, Negeri Sembilan and Perak while Kadazan/Dusun was the more prominent ethnic group with oral cancer in Sabah and the Iban in Sarawak. The gum area was the highest area for oral cancer for all the states except for Negeri Sembilan where the cheek mucosa is mostly affected (Zain et al., 1997). Smokeless tobacco use was involved in the etiology of the oral pre-cancerous and cancerous lesions. In the South Asian region, more than one-third of tobacco consumed is smokeless. Traditional forms like betel quid, tobacco with lime and tobacco tooth powder are commonly used and the use of new products is increasing, not only among men but also among children, teenagers,

women of reproductive age, medical and dental students. In India, where chewing tobacco is used with betel nuts and reverse smoking (placing the lit end in the mouth) is practiced, there is a striking incidence of oral cancer cases, which account for as many as 50% of all cancers. While cases of oral cancers are seen in patients who do not use tobacco, these constitute a very small percentage of all oral cancers. Males have this type of cancer about twice as often as females. Tobacco products, especially smokeless tobacco, are a primary cause. Females are more commonly experiencing this type of cancer as they use tobacco products (Khandekar et al., 2006).

Squamous cell carcinomas account for 90% of all oral cancers, followed by adenocarcinomas and Kaposi's sarcomas. Squamous cell carcinomas occur in all sites of the oral cavity, including tongue, lips, floor of the mouth, soft palate, and tonsils. Adenocarcinomas occur mainly in the salivary glands while Kaposi's sarcomas mainly occur in the palate. Of all the anatomic sites, the tongue and floor of the mouth account for most of these cancers. Symptoms include tender painful lesions, wounds or sores that will not heal, a lump or thickened skin, a white or red patch, loose teeth or dentures, trouble chewing or swallowing, swelling of the jaw, and sore throat (Silverman, 1999).

Inactivation of tumour suppressor genes and activation of oncogenes have been shown to play a role in the multi-step process of human carcinogenesis. Molecular genetic studies of oral squamous cell carcinoma (OSCC) have been focussed on the identification of tumour suppressor gene (TSG) loci and amplified oncogenes. Previous loss of heterozygosity (LOH) studies focussed on specific chromosome segments have shown frequent loss of alleles from 3p, 8p, 9p, chromosome 13 and 17p in head and

neck squamous cell carcinoma (HNSCC), including OSCC (Ishwad et al., 1996; Arai et al., 2002; Coon et al., 2004; Gao et al., 2007). A number of recent studies using comparative genomic hybridization (CGH) and allelotyping showed HNSCC display large and extensive genomic imbalances and that certain chromosome segment are lost more often than others. These studies confirmed the frequent deletion and LOH from 3p, 9p, 13q, and 17p, detected in more than 50% of the cases. In addition, segmental loss in 3q, 4p, 4q, 5q, 6p, 6q, 8p, 8q, 11q, 14q, 17q, 18q, and 20p have been shown in significant subsets (Weber et al., 1998).

Allelic imbalances including LOH and microsatellite instability (MSI) in malignant cells have proven useful for mapping DNA regions potentially harboring candidate TSGs. Studies on somatic LOH have led to the discovery of genes, including *RB* (Retinoblastoma gene), *p53*, *WT* (Wilms tumour), *CDKN2A* (Cycline dependent kinase number 2), *APC* (Adenomatous polyposis coli), *DPC4* (Candidate suppressor gene for pancreatic cancer), *FHIT* (Fragile histidine triad) and *PTEN* (Putative protein tyrosine phosphatase gene) (Arai et al., 2002).

LOH in chromosome 3p material, in particular 3p13-p21, 3p21-23, and 3p25, is a common genetic change shared by several types of carcinomas. Several TSGs that have been mapped to these regions include two genes that have been studied for the presence of inactivation mutations in OSCC which is *FHIT* gene in 3p14.2 and *VHL* (von Hippel Lindau) gene in 3p25-26. The finding of alterations of *FHIT* in oral precancerous lesions and SCC supports the pathogenetic role of *FHIT* in OSCC carcinogenesis. However, very little evidence for the involvement of *VHL* in OSCC could be observed (Tsui et al.,

2008). Previous study also identified a novel commonly deleted region on 3p21.3 (D3S966) suggesting that unknown TSG specific to Indian and Japanese OSCC exist at this region (Yamamoto et al., 2007).

Subsets of many types of malignancies show contraction or expansion of MSI. MSI is based on the functional impairment of one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, or *PMS2*, and germline mutations in these genes predispose for the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. Affected individuals are at an increased risk for a variety of carcinomas, not usually involving HNSCC. High-level MSI has been reported in 10% to 15% of all colorectal cancers and is based mainly on hypermethylation of the MLH1 promoter. Similarly, molecular studies have revealed that MSI and loss of heterozygosity (LOH) occur in head and neck cancer, suggesting the influence of both mutator and tumour suppressor pathways in head and neck carcinogenesis. Identification of tumours based on MMR deficiency may become important for the clinical management of the disease, as there is growing evidence from studies on colorectal cancer that such tumours have a better prognosis on one hand, but do not benefit from chemotherapy on the other (Koy et al., 2008).

1.2 Problem statement

Stage at diagnosis is the most important prognostic indicator for OSCC. Unfortunately, about 50% of these cancers are identified late (stages III or IV). If lesions are detected when they are small, localized, and treated quickly, survival rates of 70–90% can be achieved. Thus, the possibility of cure depends on the time elapsed between the first

symptom and the beginning of the treatment. Due to the effect of field cancerization, patients with OSCC may suffer recurrences after the treatment or develop second malignant tumours at various sites (Morelatto et al., 2007).

Radical surgery is the primary treatment of OSCC. High-stage disease often requires postoperative adjuvant radiotherapy to eliminate all residual disease and is therefore more distressing for patients and, unfortunately, less successful in survival when compared to treatment of low-stage disease, whereas patients with high-stage tumours of larger size and higher metastatic potential have only a 30% chance of survival at 5 years, patients with low-stage cancer have an 80% to 90% chance of survival. A high degree of clinical expertise is required to detect OSCC, and this is not widely available. The development of detection methods (such as blood tests) that can act as a surrogate for a highly skilled clinical examination would allow detection of OSCC at an early stage as well as monitoring response to treatment and helping to identify residual disease after treatment. This would significantly improve survival as treatment of early stage disease has been shown to be more successful (Tiziani et al., 2009).

Early detection and even screening high risk populations of OSCC and precursor lesions is an attractive strategy to reduce the suffering of OSCC, and thus scientists engage to find efficient diagnosis and preclinical screening approaches. For decades, numerous publications on the use of oral cytology or histological manifestations as a diagnostic approach have been reported. In recent years, considerable progress has been made in understanding the genetic and proteic basis of OSCC carcinogenesis and a number of

specially expressed genes and proteins are proposed as biomarkers for clinical diagnosis (Yan et al., 2008).

1.3 Justification of the study

Studies on microsatellite alterations (LOH and MSI) have demonstrated genomic instability reflecting defective mismatch repair system and presence of putative tumour suppressor genes. Molecular markers allow intermediate measurement of the outcome of the therapy as molecular changes occur before histological changes and are essential in the diagnosis and management of patients with oral cancer. The selection of microsatellite markers D3S192, D3S647, D3S966, D3S1228, D3S659 located on chromosome 3p is based on their highly informative nature in OSCC (Arai et al., 2002).

The incidence of OSCC varies in different parts of the world and the epidemiological difference and frequencies of MSI and LOH in the different population may be reflected at the molecular level. Several studies using different sets of microsatellite markers detected MSI frequencies at great diversity, ranging from 2% to 60% (Koy et al., 2008). Because of limited information on microsatellite alterations in OSCC in a Malaysian populations, the present study will provide a basis on the understanding of the different frequencies in which these microsatellite occur, which in turn will provide an insight into the molecular differences persistent in the Malaysian population. This will help in unravelling the genetic causes underlying the OSCC in the Malaysian population which will target to new interventions and clinical care directed at the molecular changes of cancer.

1.4 Objectives

1.4.1 General objective

The purpose of this study is to determine the percentage of microsatellite alterations on chromosome 3p in OSCC in a Malaysian population.

1.4.2 Specific objectives

1 To determine the percentage of LOH of the five microsatellite markers on chromosome 3p in OSCC in a Malaysian population.

2 To determine the percentage of MSI of the five microsatellite marker on chromosome 3p in OSCC in a Malaysian population.

3 To associate the percentage of LOH with the histopathological grading in the cancer tissue.

4 To compare the MA between different markers selected on chromosome 3p in the cancer tissue.

1.5 Research hypothesis

Microsatellite alterations on chromosome 3p are associated with development of OSCC in a Malaysian population.

CHAPTER 2

LITREATURE REVIEW

2.1 Oral cancer

Oral cancer is a part of a group of cancers called head and neck cancers. Oral cancer includes tumours of the lip, tongue, floor of the mouth and pharynx (Canto and Devesa, 2002). OSCC accounts for 90% of all oral cancers, followed by adenocarcinomas and Karposi's sarcomas (Syme et al., 2001). OSCC are thin, flat cells that look like fish scales when observed under the microscope. Of all the anatomic sites, the tongue and floor of the mouth account for most of these cancers (Figure 2.1).

2.1.1 Prevalence and incidence of oral cancer

OSCC is the sixth most common cancer reported worldwide with estimated 400,000 new cases annually (Seeram et al., 2011). Incidence rates are more than twice as high in man when compared to woman. Higher prevalence was found in Asian populations, mainly in South Asian countries including India and Pakistan where it is the most common cancer in males and the second most common cancer in both sexes (Iamaroon et al., 2004). In Malaysia, mouth cancer is the 20th most common cancer for females and 28thfor males of overall cancer incidence per 100,000 populations in the year of 2006 (Ministry of Health, 2006). Oral cancer among the Indian males and females accounts for 2.5% and 7.3% respectively of all cancers. It can be ranked as 14th and 4th most

common cancers for the Indian males and females respectively. Hospital based data from different hospitals in Malaysia has shown that oral cancer is highest among ethnic Indians and other Bumiputras (the indigenous people of Sabah and Sarawak) followed by the Malay and Chinese (Ministry of Health, 2006).

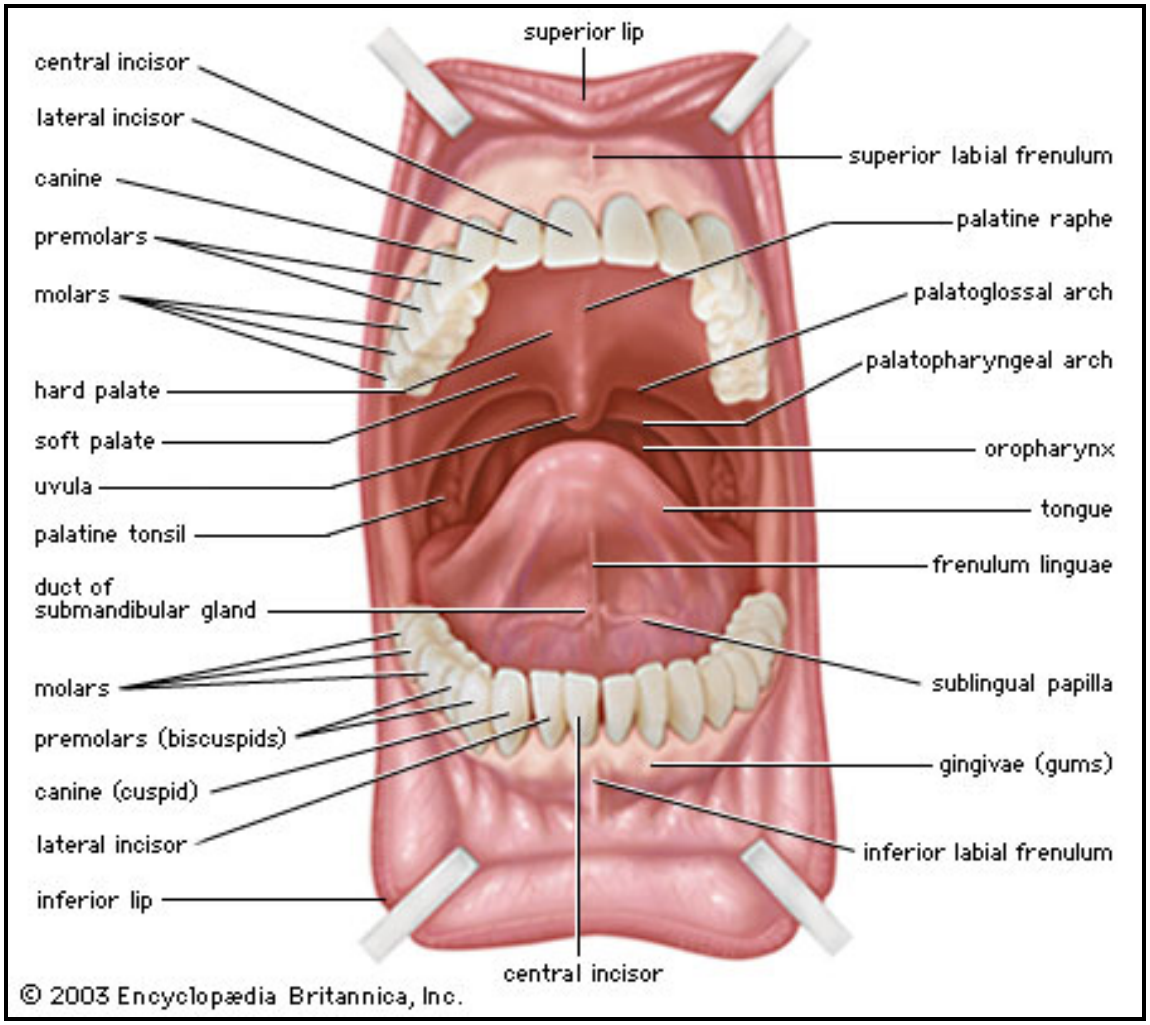

Figure 2.1: Mouth anatomy (http:www.boddunan.com/health-a-fitness/66-diseases/1850-what-is-mouth-cancer.html).

2.1.2 Risk factors of oral cancer

Smokeless tobacco and alcohol use are the major causes of oral cancer. People who smoke and drink have 10 times more risk to get oral cancer than people who do not smoke and drink and 100 times greater in people who smoke and drink heavily (Lippman et al., 2005). The risk of oral cancer associated with smoking is both dose and duration dependent while smoking cessation leads to a fall in risk. Other risk factors are betel quid chewing, actinic radiation (sunlight exposure), immune system deficiencies, viral infections (Human Papilloma Virus), nutritional deficiencies, and certain oral condition (Canto, 2002).

The incidence of oral cancer is age related, which may reflect time for the accumulation of genetic changes and duration of exposure to initiators and promoters (chemical and physical irritants, viruses and hormonal effect), cellular aging and decreased immunologic surveillance with aging. About 95% of all oral cancers occur in persons over 40 years old, and the average age at the time of diagnosis is 60 (Burket, 2003).

Ethnic background is known to influence many types of cancers. For example, in Malaysia oral cancer in Indians is increasing at faster rate than in Chinese and Malay. The chronic use of betel quid (paan) in the mouth among Indian people has been strongly associated with an increased risk for oral cancer (Johnson, 2001). The quid typically consists of a betel leaf that is wrapped around a mixture of areca nut and slaked lime, usually with tobacco and sometimes with sweeteners and condiments. The slaked lime results in the release of an alkaloid from the areca nut, which produces a feeling of

euphoria and well-being in the user. Betel quid chewing often results in a progressive, scarring precancerous condition of the mouth known as oral submucous fibrosis. This suggests that genetic factors, differences in habits and lifestyle are strongly implicated. Variation in incidence rate among ethnic groups may be influenced by differences in exposure to carcinogenic initiator or promoter (Avon, 2004).

2.1.3 Oral Carcinogenesis

Oral carcinogenesis is a multistep process requiring the accumulation of multiple genetic, epigenetic and metabolic alterations, influenced by a patient's genetic predisposition as well as by environmental influences, including tobacco, alcohol, chronic inflammation, and viral infection (Lippman et al., 2005). OSCC development usually involves normal oral mucosa changing to oral dysplasia and changing to invasive cancer (Figure 2.2). Oral dysplasia may progress to OSCC over time and therefore can be considered as premalignant lesions. Dysplasia can be detected only by taking a biopsy of the lesion. Examining the dysplastic cells under a microscope (histology) indicates how severe the changes are and how likely the lesion is to become cancerous (Epstein et al., 2007).

The two most common kinds of premalignant lesions in the oropharynx are leukoplakia and erythroplakia (Figure 2.3). Leukoplakia is a white or whitish lesion of the oral mucosa that cannot be clinicopathologically characterized as any other definable lesion. These lesions are usually caused by smoking and chewing snuff. About 8% of leukoplakias are cancerous at diagnosis or will become cancerous within 10 years if not

treated. Erythroplakia is a raised, red patch and may bleed if scraped. Erythroplakia is generally more severe than leukoplakia and has a higher chance of becoming cancerous over time (Waal et al., 1997).

Squamous cell carcinoma is a common type of cancer account for more than 90% of all cancers (Figure 2.3). These cancers start in the squamous cells, which form the surface of many layers of the mouth and pharynx. They can invade deeper layers below the squamous layer. When oral cancer spreads, it usually travels through the lymphatic system. It can also spread to other parts of the body. For example, if oral cancer is spread into the lungs, the cancer cells in the lungs are actually oral cancer cells. Doctors sometimes call the new tumour "distant disease or metastatic" (Harrison, 2009).

Molecular mechanisms underlying oral carcinogenesis remain largely unknown. Damage affect many chromosomes and genes, particularly TSGs and oncogenes and it is the accumulation of such genetic damage and the consequent disturbed cell growth and control, which in some instances appears to lead to carcinoma. At the moment of cancer detection, when malignant growth becomes macroscopically detectable, many steps towards a mature cancer have already taken place. In normal cells, genetic mutations will not lead to cancer, since DNA repair mechanisms and cell cycle checkpoints are actively protecting cells from becoming malignant. When mutations accumulate within these protective systems, a “mutator phenotype” develops and an important step towards cancer has been set (Kaufmann and Paules, 1996).

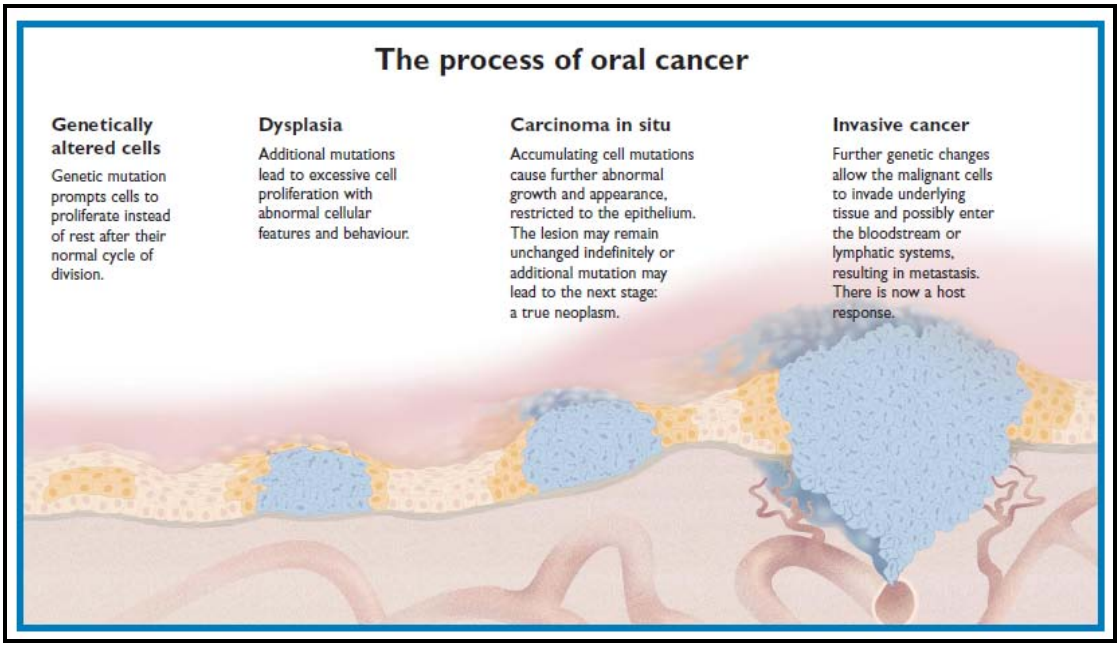

Figure 2.2: Oral cancer development (http://oralcancerfoundation.org).

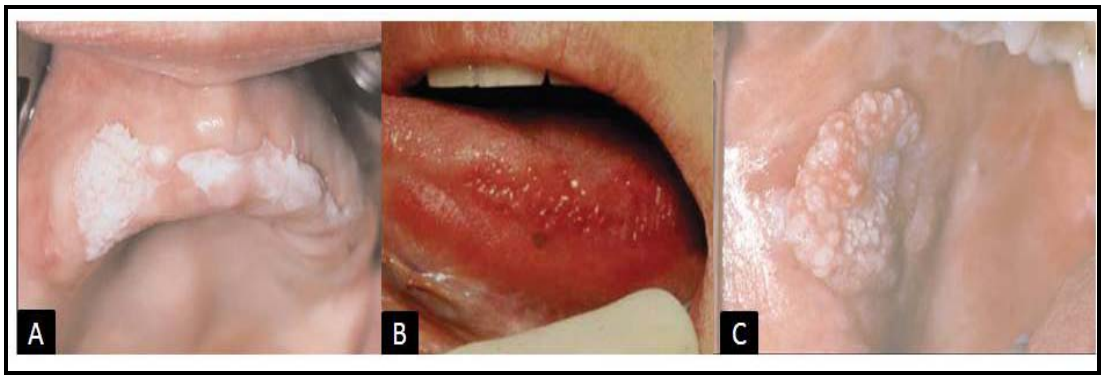

Figure 2.3: Oral cancer and precancerous lesion. (A) Leuokoplakia seen as a white lesion (B) Erythroplakia of the ventral tongue seen as a well demarcated red patch. (C) Squamous cell carcinoma seen as exophytic, papillary mass of the buccal mucosa (Bouquot et al., 2010).

2.1.4 Genetic and epigenetic alterations of oral cancer

The accumulation of genetic and epigenetic alterations drives the progression of normal cells to dysplastic stages and to invasive cancer (Figure 2.2). Mutational and gene expression analysis of known TSGs and oncogenes in early tumourigenesis has provided insight to the role of these genes in cancer progression (Holmes et al., 2007). In recent years, considerable research has been made to identify the candidate TSGs that lead to oral cancer. Accumulation of genetic and epigenetic alterations include alteration of TSG such as *FHIT* (LOH at chromosome region 3p14), *p16* (LOH at 9p21 and *p16* promoter hypermethylation), *VHL* (mutation and LOH at 3p25) and *p53* (mutation and LOH at 17p) (Arai et al., 2002, Sakamoto et al., 2004, Lippman et al., 2005, Yamamoto et al., 2007).

p53 TSG on chromosome 17 is responsible to produce 53kDa nuclear phospoprotein, that binds to DNA and suppress cell growth and division. If cell repair is impossible, *p53* triggers apoptosis (programmed cell death). Mutation or LOH at *p53* region will alter *p53* protein that will predispose to develop cancer. *p53* are most common genetic alterations in many malignancies, including oral cancer (Conde et al., 1999). Previous studies have reported that inactivation of *p53* gene is associated with the development of oral cancer (Huang et al., 1999). Aberration in *p53* is an early event of oral carcinogenesis but it is not the only gene that is responsible and many other molecular events occur.

Mutation and LOH of *VHL* gene located at 3p25 are frequently seen in oral cancer. The *VHL* gene encodes 4.7kb mRNA, which is widely expressed in both fetal and adult tissue, such that expression of *VHL* transcript and protein is ubiquitous and not restricted to those organs affected by *VHL* tumours. In normal condition, *VHL* gene will prevent cells from growing and dividing too rapidly or in an uncontrolled way. In contrast, mutation in *VHL* gene will promote progression of oral cancer (Richards, 2001; Lippman et al., 2005)

p16 also known as CDKN2A, is a tumour suppressor protein, which in humans is encoded by the *CDKN2A* gene. *P16* plays an important role in regulating the cell cycle, and mutations in *p16* increase the risk of developing a variety of cancers. Inactivation of *p16* gene by CpG methylation is a frequent event in oral epithelial dysplasia. Latest study by Cao et al., (2009) reported that *p16* methylation can lead to malignant transformation of oral epithelial dysplasia and Vairaktaris et al., (2007) also reported that inactivation of *p16* occurs at the early stage of oral mucosal dysplasia in the multistep process of oral tumourigenesis. Therefore, *p16* may be considered as a useful prognostic marker for the progression of oral cancer and is a potential biomarker for prediction of prognosis of mild or moderate oral epithelial dysplasia.

Alteration in *FHIT* gene has been reported to be associated with oral carcinogenesis. LOH at *FHIT* region (3p14.2) have been found in various human malignancies including HNSCC, lung cancer and renal cell carcinoma (Tanimoto et al., 2000; Kujan et al., 2006). This gene, a member of the histidine triad gene family, encodes a diadenosine 5',5'''-P1,P3-triphosphate hydrolase involved in purine metabolism. The gene

encompasses the common fragile site FRA3B on chromosome 3, where carcinogen-induced damage can lead to translocations and aberrant transcripts of this gene. In some tumours, particularly those associated with environmental carcinogens, alterations in the *FHIT* gene occur quite early in the development of cancer. In other cancers, *FHIT* inactivation seems to be a later event, possibly associated with progression to more aggressive neoplasias (Croce et al., 1999).

2.1.5 Management of oral cancer

Oral cancer is lethal disease with 5 years survival of less than 50%. Death owing to distant metastasis, once considered to be a rare feature, is now more common as a result of better loco regional control (Canto, 2001). Early detection is the main factor for better prognosis. Generally, oral cancers are curable when diagnosed and treated in localized stages. Clinicians' knowledge of the early signs and symptoms of oral cancer is important for diagnoses and appropriate referral. To accurately detect a lesion in the early stages, general clinician must be familiar with where to look and what to see in oral cavity. The past few decades have seen an increasing knowledge in oral carcinogenesis as well as technology advances in the diagnosis of oral cancer. These include epidemiology of oral cancer, detection and early diagnosis of oral cancer, and treatment of patients (radiotherapy, chemotherapy, gene therapy and surgery) (Canto, 2001).

2.1.5.1 Diagnosis of oral cancer

Detection and early diagnosis of oral cancer involve both individual and professionals. Individual is responsible to detect when the first symptom appear. Dentists are the provider of choice to perform oral cancer examinations but unfortunately majority of them do not visit a dentist in a given year (Goodman et al., 1995). Thus, other health care providers must assume more responsibility to ensure that the public receive oral cancer examinations on a routine basis. Primary care physicians should know that targeting those at high risk is a viable and cost-effective intervention for oral cancer when performed as part of routine practice. Equally important, members of the public need to know that an examination for oral cancer is available and that they can request one routinely. Thus, both health care providers and the general public need to increase their knowledge and change their behaviors or practices (Horowitz et al., 1996).

The prognosis of oral cancer is conditioned by several variables such as histopathological variety, degree of tumour differentiation, extension and localization of the primary tumour, degree of invasion of neighboring structures, presence of metastatic regional lymph nodes, presence of distant metastases, the choice of therapeutic modality, and the general health status of the patient. Once clinical signs of oral cancer are seen, a biopsy will be taken, followed by histopathological examination. Primary care providers usually refer to a specialist for the biopsy and further follow up, although several primary care providers may choose to biopsy the lesion. A histopathological report confirming invasive oral cancer will usually trigger the treatment mechanism. However, tumours that appear similar morphologically and histologically will show varying

responses when treated in identical way. Thus, the use of a new technology to diagnose oral cancer is needed (Brandizzi et al., 2008).

While the biopsy is still required for a definite diagnosis, today several non-invasive methods are commercially available to aid clinicians in the diagnosis of oral cancer. Some of the most common methods include a method using a brush biopsy that collects oral cells and examining them under the microscope for signs of malignant transformation. The second methods which is a chemiluminescent method that uses a light followed by tolouidine blue application for vital tissue staining. The third method is a method that uses a device emitting a blue light to examine differential tissue fluorescence. While adjunctive techniques that are non-invasive usually lead to a high rate of false positive findings, they can offer significant aid in healthcare professionals who are highly trained with the specific method to spot high risk lesions that are either sub-clinical or at their very early phases (Donnell, 2008). However, the fact that they are used by only a fraction of dentists in the United States presents practical and logistical difficulties during referrals. Anecdotal reports exist about dentists who identify lesions and refer them to specialists for further evaluation, only to have the specialists dismiss the case due to lack of ability to spot the same lesion without the use of the same technology at the specialists office. A systematic intervention is needed to assess the scientific and clinical validity of a network of healthcare professionals all using standard same methods, applied in a community setting (Donnell, 2008).

2.1.5.2 Treatment of oral cancer patients

Treatment of the patients involves radiotherapy, chemotherapy and surgery. Contemporary radiotherapeutic treatment encompasses new forms of radiation and the application of sophisticated computerized methods to enhance the therapeutic effectiveness with an important reduction in the irradiation of normal surrounding tissues. This has led to an increased therapeutic dose in the tumour site and a decreased severity of radiation-induced injuries. Methotrexate was surpassed by platinum based agents with or without 5-Fluorouracil. Adjuvant and neoadjuvant schemes coupled with pre- or post-operative radiotherapy starting from the late 80s showed a distinct survival benefit over radiotherapy alone (Fuller et al., 2010). This major breakthrough was followed by the introduction of taxanes and the development of molecular targeted therapies which, during the last 5 years, have revolutionized the concept of chemo-radiation. Induction chemotherapy and chemo-radiation coupled with epidermal growth factor receptor (EGFR) antagonists have proved to offer a survival benefit to patients with locally advanced or recurrent squamous cell carcinoma (Blackhall et al., 2005). The revolution in the surgical treatment of oral/head and neck cancer was the introduction of reconstructive techniques with both pedicled locoregional flaps and free tissue transfer which allow for safer and wider resections with adequate disease free margins and functional reconstruction of the created surgical defects (Clayman et al., 1997). There have been major changes in the surgical management of both clinically negative and clinically positive neck patients, as well as in the management of the mandible, especially where there is early mandibular invasion. However, modification of

radiotherapy, chemotherapy and surgery has not improved the survival rate of the patients (Clayman et al., 1997).

Advances in molecular biology and genetics, and the human genome project, led to an explosion in deciphering oncogenes, TSGs and other genes, and their biological role in oral carcinogenesis. Gene therapy is one of the approaches of new treatment for oral cancer patients. Gene therapy can be defined as gene transfer for the purpose of treating human disease (Xi and Grandis, 2003). This includes the transfer of new genetic material as well as the manipulation of existing genetic material. This holds true especially for cancer cells, where dominantly activated oncogenes can be targeted. The transfer of genetic material may occur *in vivo* (where the gene is introduced into the body) or *ex vivo* (where a tumour is removed, the genetic materials delivered, and the cells are then re-introduced into the patient). The *ex vivo* approach has not been utilized in oral cancer because superficial lesions usually lend themselves to the direct injection of genetic material (Saraswathi et al., 2007).

2.2 Microsatellite

Microsatellite analyses include LOH and MSI, which is one of the techniques used to detect possible regions of TSGs and oncogenes. Microsatellites of DNA are tandem repeats of 1–6 nucleotides found scattered throughout the human genome. Microsatellites (also called simple sequence length polymorphisms: SSLPS**;** and short tandem repeat polymorphisms: STR) can be defined as tandem arrays of short stretches of nucleotide sequences, usually repeated between 15 and 30 times. Microsatellites

belong to the family of repetitive non-coding DNA sequences, which can be classified as follows. (i) Satellite sequences: arrays with repeat sizes ranging from 5 to 100 bp, characteristically organized in clusters up to 100 mega bases (Mb). (ii) Minisatellite sequences: arrays with repeat sizes of 15-70 bp which range in size from 0.5 to 30 kilobases (kb). (iii) Microsatellite sequences: arrays with a repeat size of 2-6 bp, highly variable in size but ranging around a mean of 100 bp. Microsatellites are found in the euchromatin and allele sizes in populations characteristically exhibit multiple size classes distributed about the population mean. Microsatellites have various applications; these include genome analysis, allelic imbalance analysis, population studies and forensic application (Koreth et al., 1996).

2.2.1 Microsatellite instability

Microsatellite instability in cancer cell is defined as a change that occurs in the DNA in which the number of repeats of microsatellites is different than the number of repeats that was in the DNA when it was inherited. The frequencies at which changes in repeat number occur at microsatellite loci are much higher than normal mutation rates, involving a frequent change in repeat number by one or two repeats at a time, and rare large changes in repeat number, representing two distinct classes of mutation at microsatellite loci. This slippage synthesis model can explain the relation between defective DNA repair and MSI seen in some disease states (Figure 2.4). It was initially thought that repeat sequences possessed a functional role in the genome, either directly via a role in gene regulation or indirectly as hot spots for recombination, their mutagenic

potential enhancing the long-term evolutionary potential of the species (Koreth et al., 1996).

Microsatellite instability is mostly found in association with deficiencies in DNA mismatch repair systems and was first described in hereditary non-polyposis colon cancer (HNPCC) patients. MMR genes that are related with this cancer are *MLH1*, *MSH2*, *MSH6* and *PMS2*. The colorectal tumours are often classified into MSI-high (changes in≥2 markers), MSI -low (exhibiting instability at one marker) or MSI-stable exhibiting no changes in the selected loci. MSI-high has been reported in 10%-15% of all colorectal cancers and is based mainly on hypermethylation of the *MLH1* promoter (Robinson et al., 2007). Several studies using different sets of microsatellite markers detected MSI percentage at a great diversity ranging from 2%-60% (Koy et al., 2008). In oral cancer, alterations in microsatellite have been reported with variable percentage while the majority of studies show low percentage. Therefore, the importance of MSI in oral cancer is not well established, because of lack of standardized and comparable parameters among the so far published studies. Moreover, none of the MSI tumours has been found to be associated with germline mutation (Koy et al., 2008).

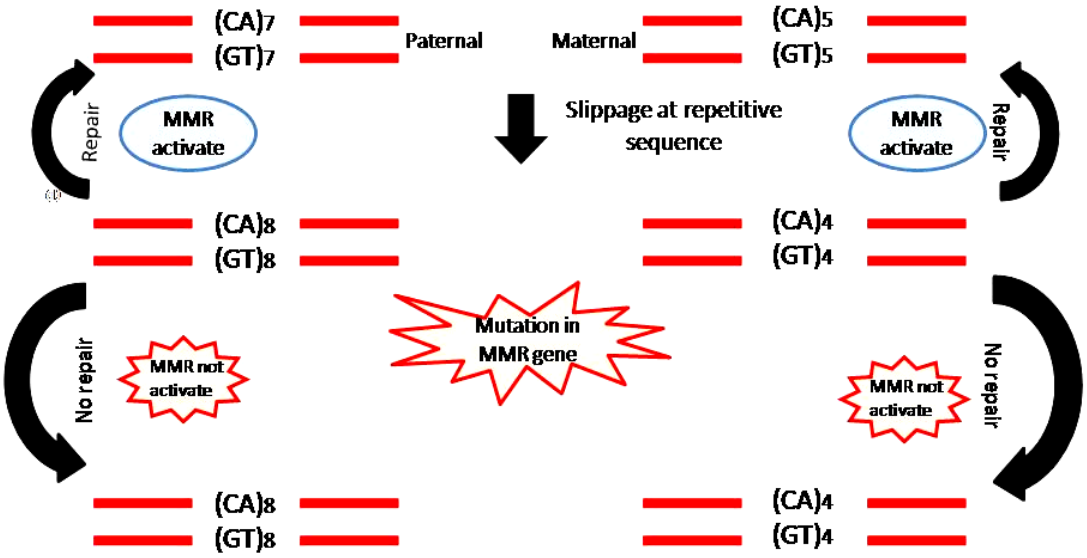

Figure 2.4: The effects of microsatellite instability on (CA)n dinucleotide tandem repeats. Repetitive sequences, represented by CA repeats, can slip either at the parental DNA or newly synthesis daughter DNA strand. This slippage can be repaired by mismatch repair (MMR) system. However, mutation in MMR gene make MMR enzyme failed from identifying this error and will leave this strand uncorrected. Consequently, loss of the MMR system leads to development of cancer (http://phg.mc.vanderbilt.edu/content/ezlod).