CYCLIN D1 AMPLIFICATION IN TONGUE AND BUCCAL MUCOSA SQUAMOUS CELL CARCINOMA

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ABSTRACT

Introduction

Oral cancer is a significant health problem worldwide with almost 300,000 new cases are diagnosed each year. Despite the numerous studies done, and even with the best treatment option utilized, more than 50% of patients with oral cancer will experience relapse. In search for better options for prognostication, researches are now focusing on the molecular biology of cancer, for instance in search of reliable tumor markers. Among the markers reported in the literatures, Cyclin D1 is actively studied protein. Cyclin D1 regulates the cell cycle progression by forming a complex with different cyclin dependant kinase. Dysregulation of cyclin D1 can result in loss of normal cell growth and tumor development. The aim of this study is to determine and compare the amplification of Cyclin D1 in buccal mucosa and tongue oral squamous cell carcinoma(OSCC) and to associate its amplification in buccal mucosa and tongue OSCC with tumor depth, tumor front, histopathological grading, pathological tumor size, lymph node status, TNM staging and survival rate.

Materials and methods

The study samples were paraffin-embedded OSCC surgical specimens obtained from the archives of the Department of Oral Pathology, Oral Medicin and periodontolgy and Oral Pathology Diagnostic Laboratory. Fifty samples of patients with primary OSCC of buccal mucosa and tongue were included in the study. The sociodemographic and clinical data were obtained from the Malaysian Oral Cancer Tumor and Database System coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC), University of Malaya. There were 31(62%) female and 19(38%) male with the overall age ranging from 26 to 94 years with a mean age of 60years.

The OSCC samples were from 44(68%) Indians, 10(20%) Malays and 6(12%) Chinese. The fluorescent-in-situ hybridization (FISH) technique was used to detect the amplification of Cyclin D1 using the Vysis protocol. Fluorescence evaluation of Cyclin D1 was performed using the image analyzer where the Cyclin D1 amplification signal appears as a small spot. At least 200 nuclei were scored using a 100X objective in each defined histological area, and each nucleus was assessed for the chromosome copy number. Statistical correlations of Cyclin D1 and certain clinicopathological parameters of OSCC were analyzed using the chi-square method or Fisher's exact test

Results

The present study found positive amplification of cyclin D1 in 72% (36) of OSCC. Detection of positive amplification for cyclin D1 was observed in 88% (22) and 56% (14) of the tongue and buccal mucosa OSCC respectively where the difference was statistically significant($p=0.012$). There was a significant correlation between Cyclin D1 positivity and ethnicity for the OSCC of the buccal mucosa ($p=0.037$); larger pathological tumor greatest dimension (pT) ($p = 0.019$), higher pTNM stages ($p=0.014$), tumor depth $\geq$ 5mm in tongue cases ($p<0.001$) and survival rate ($p=0.009$) for overall SCC cases and ($p<001$) for buccal mucosa SCC cases.

Conclusion

There is a significant correlation between amplification of Cyclin D1 with tumor depth and size of the tumor for tongue SCC; ethnicity and survival rate for buccal mucosa SCC .

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LIST OF ABBREVATIONS

CCND1	Cyclin D1
CDK4\6	Cyclin dependent kinases 4 and 6
DAPI	4',6'-diamidino-2-phenylindole
EBV	Epstein-Barr virus
FISH	Florescence in Situ Hybridization
H&E	Hematoxylin and Eosin
FITC	Fluorescein Isothiocyanate
HIV	Human immunodeficiency virus
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human papilloma virus
IHC	Immunohistochmistry
MOCTDBS	Malaysian Oral Cancer Tumor and Database
OCRCC	Oral Cancer Research Coordinating Center
OSCC	Oral squamous cell carcinoma
pN	Pathological lymph node metastasis
pT	Pathological tumor greatest dimension
pTNM	Pathological TNM stage
Rb	Retinoblastoma
SPSS	Computer program used for statistical analysis
TNF	Tumor necrosis factor
WHO	World Health Organization
XP	Xeroderma Pigmentosum

CHAPTER ONE:

INTRODUCTION AND OBJECTIVES

1.1. Introduction:-

Oral cancers are defined as neoplasm involving the oral cavity, which begin at the lips and end at the anterior pillar of the fauces (it may be primary lesion originating by metastasis from a distant site of origin or by extension from a neighboring anatomic structure) (John, 2007).

Oral squamous cell carcinoma (OSCC) is the most common histopathological type of oral cancer, which represent approximately 91% of all oral malignancies worldwide and in Malaysia. Well-differentiated SCC is the most commonly encountered histological variant (Silververg 1995, Ng & Siar. 1997). Its frequency has been described as directly related to alcohol consumption and smoking (Stephen 2001).

Initiation, promotion, and progression are the major phases of multistage process of oncogenesis (Nowell 1976 , Weinberg 1989, Farber 1980). Normal cells are transformed into malignant cells by mutations in genes (oncogenes) that regulate cell cycle progression and mutations in tumor suppressor genes. These multiple events lead to uncontrolled proliferations of abnormal cells and the development of cancers (Weinberg 1989, Vogelstein et al.1993, Stanbridge et al. 1990, Toshiyas et al. 1995).

The activation or amplification of proto-oncogenes, such as members of the epidermal growth factor receptor family, or cyclin D1, results in cellular proliferation

(Toshiyas et al. 1995, Yamamoto 1986, Xiong et al. 1992). Inactivation of tumor suppressor genes also enhances tumor progression, including members of the retinoblastoma gene family, cyclin-dependent kinase inhibitors, and p53 (Toshiyasu et al. 1995, Weinberg 1991).

Evidence from the literature suggests that there is marked, inter-country variation in both the incidence and mortality from oral cancer. There is also growing evidence of intracountry ethnic differences, mostly reported in the UK and USA. These variations among ethnic groups have been attributed mainly to specific risk factors, such as alcohol and tobacco (smoking and smokeless), but dietary factors and the existence of genetic predispositions may also play a part. Variations in access to care services are also an apparent factor. The extent of ethnic differences in oral cancer is masked by the scarcity of information available. Where such data are accessible, there are clear disparities in both incidence and mortality of oral cancer between ethnic groups.(Scully, & Bedi, 2000)

Since the early 1920's a variety of staging systems have been developed with many focused on a specific type of cancer. For example, the Duke's system for colorectal disease and Ann Arbor classification for lymphomas. The most widely used staging system in the world is the TNM (i.e. Tumor, Node, Metastases) system which is now in its sixth edition (Grunfeld, E. 2005).

Among factors that affect the prognosis of oral cancer is staging of the cancer. However, it was found that some tumors with similar clinical staging still show

different growth patterns and prognosis (Platz et al. 1983; Platz et al. 1985). This is because, the biological characteristic of tumors are often variable, resulting in divergent clinical disease courses despite identical staging. Many researches aim to identify molecular and biological prognostic factors in order to predict clinical aggressiveness (Greene et al. 2002).

Cyclin D1 proto-oncogene is an important regulator of G1 to S-phase transition in numerous cell types from diverse tissues. Binding of cyclin D1 to its kinase partners, the cyclin dependent kinases 4 and 6 (CDK4/6), results in the formation of active complexes that phosphorylates the Retinoblastoma tumor suppressor protein (Rb). Hyperphosphorylation of Rb results in the release of Rb-sequestered E2F transcription factors and the subsequent expression of genes required for entry into S-phase. More recently, cyclin D1 has also been shown to act as a cofactor for several transcription factors. Initial studies indicated that cyclin D1 is localized predominantly in the nuclei of asynchronously growing cells. (Baldin 1993)

During cell cycle progression, levels of the cyclin D1 begin to rise early in G1, prior to its rapid nuclear export and degradation within the cytoplasm. Interestingly, the nuclear export and/or degradation of cyclin D1 is required for S-phase progression as failure to remove the cyclin results in G1 arrest. (Baldin 1993, Guo et al. 2005)

There are various approaches to evaluate cyclin D1 deregulation such as Southern blot hybridization, polymerase chain reaction (PCR), and immunohistochemistry.

Most investigators reported cyclin D1 amplification through DNA transfer and hybridization techniques such as dot, slot, and Southern blotting. These methods require a sufficient quantity of DNA. However, specimens from OSCC patients are generally limited in size to obtain adequate amounts of the tumor tissue for molecular genetic analysis other than histologic diagnosis.

Therefore, it may be difficult to perform these methods on many OSCC specimens. Although PCR is suitable for a small amount of DNA, this method has the problem of normal cell contamination. Genetic abnormalities, such as amplifications, deletions, and chromosomal rearrangements cannot be estimated by immunohistochemical staining. Conversely, FISH analysis requires very little tumor tissue and the method is rapid and does not involve radioactivity.

For rearrangements that do not involve genomic imbalances, such as balanced chromosome translocations and inversions, the use of CGH is limited. In addition, whole-genome copy number changes (ploidy changes) cannot be detected. Furthermore, CGH provides no information about the structural arrangements of chromosome segments that are involved in gains and losses.(Ryan 2010)

Although FISH technique cannot detect point mutation, genetic aberrations can be identified easily, even when only few neoplastic cells are present in the specimen.

Therefore, FISH technique may be well suited for use in genetic analysis of primary OSCC specimens (Miyamoto R 2002).

1.2. Aims and objectives:-

The aim of this study is to explore the feasibility of Cyclin D1 as a prognostic marker using Fluorescent In Situ Hybridization method.

The objectives of this study are as follow:

1. -To determine and compare the amplification of Cyclin D1 in tongue and buccal mucosa SCC by using Fluorescent In Situ Hybridization method.
2. -To associate the amplification of Cyclin D1 in tongue and buccal mucosa SCC with age, gender, ethnicity, pTNM, lymph node status, greatest tumor dimension, pattern of invasion and modified Broder's grading.
3. - To associate the amplification of Cyclin D1 in tongue buccal mucosa SCC with survival rate.

CHAPTER TWO:

LITERATURE REVIEW

2.1. Etiology and risk factors:

The cause of OSCC is multifactorial which involve both extrinsic and intrinsic factors. Extrinsic factors implicated include tobacco smoke, alcohol, syphilis, sunlight, oncogenic viruses and candidal infection. Intrinsic factors considered are systemic or generalized states, like malnutrition, immunosuppression and involvement of oncogenes and tumor-suppressor genes. Lichen planus and oral submucosal fibrosis are conditions associated with increased risk of intra-oral malignancy. Despite the fact that a premalignant lesion such as epithelial dysplasia is recognized as one of the risk factor, but many oral cancers do not go through a premalignant stage (Cawson 2002).

2.1.1. Genetic and familial factors:

In a study by Prime et al. which reviewed the role of inherited cancer syndromes and their association with OSCC, Li-Fraumeni syndrome (LFS) was suggested as a predisposing factors to OSCC. However, Patrikidou et & Haris (2001) disagrees with Prime et al. (2001) stating that there was no substantial evidence in the literature to associate the syndrome with OSCC. However, both reached the conclusion that the ongoing evaluation of malignancies in LFS patients is important.

Genetic predispositions to cancer in other inherited cancer syndromes are more clear-cut for example, Xeroderma Pigmentosum (XP) where there is an increased risk of basal and squamous cell carcinoma in skin (Sancar 1996, Chidzonga 2005).

2.1.2. Ultraviolet radiation:

Ultraviolet radiation was found to be a risk factor in many cases of lip cancers and the high incidence rates of lip cancer is associated with increase exposure to sunlight ultraviolet radiation (Ogden et al. 2000) which is well supported by studies from Finland (Warnakulasriya et al. 1994), Sweden (Horowitz 2000) and India (Dodds et al. 1994). In general, fair-skinned people are more predisposed to ultraviolet radiation-related cancer (De Visscher et al. 1998).

2.1.3. Tobacco:

There is no doubt that tobacco is the traditional risk factor for oral cancer in adults. It is the most potent toxin and major carcinogen to the human body causing both initiation and promotion of oral cancer whether smoked, chewed or snuffs. Its extensive devastating effects on almost every part of the human body either physically or psychologically are highlighted by the World Health Organization in its publication "The tobacco health toll" (WHO 2005). The main reason for prolonged usage of tobacco is nicotine addiction, despite all the well-known adverse effect (Warnakulasuriya 2005). DeMarini (2004) had reviewed the genotoxicity of tobacco smoke extensively. The ability of smokeless tobacco in delivering its nicotine is unquestionable (Ayo-Yusuf Swart TJP & W Pickworth 2004, Levy , et al. 2004, Rodu et al. 2004).

2.1.4. Quid chewing:

Quid chewing is a habit predominantly seen as an eastern culture and has been found to be the most important factor associated with transformation of normal mucosa epithelum to SCC. High prevalence was recorded in countries like India (Balaram et al. 2002), Pakistan (Mahazir et al. 2006), Taiwan (Ko et al. 1995) and Cambodia (Pickwell et al. 1994). In the western countries, the habit is more commonly practiced by the migrant communities from the eastern countries (Gupta et al. 2004).

2.1.5. Alcohol:

Alcohol consumption and tobacco smoking have synergistic effect in increasing the risk of OSCC (Ko 1995). Fransceschi (1999) had also demonstrated the increased risk of OSCC with an increase in alcohol consumption if the level of smoking remained constant. This trend was further supported by Hindle et al. (2000), Petti S (2005) and Altieri et al. 2004 and the risk has been demonstrated to be dose dependent (Franceschi et al. 1999, Schildt 1998). The role of different types of alcoholic beverages in OSCC remained controversial (Burim 2004). However, Altieri et al. (2004) concluded that despite the controversy, ethanol is the main component that contributes to the increased risk.

While tobacco smoking is more associated with soft palate cancers, alcohol drinking is more associated with cancer of the floor of the mouth and tongue (Boffeta et al.1992). Increased alcohol consumption has contributed to rise in oral, tongue, pharyngeal and esophageal cancer in Denmark (Moller 1989).

2.1.6. Infection:

2.1.6.1. Viral Infection:

Human papilloma virus (HPV) appears to be significant independent risk factor for OSCC. Human papilloma virus infection is associated with 3-6 times increased risk of OSCC independent of exposure to tobacco or alcohol consumption (Smith et al. 1998, Miller et al. 2001).

Epstein-Barr virus (EBV) has been shown to be more prevalent in OSCC than in normal mucosa but the role of EBV in OSCC is still unclear (Sand , et al. 2002). In the same study, Sand et al. (2002) also showed that smoking, alcohol use or age did not seem to be a risk factor for EBV infection. Kobayashi et al. (1999) had in their study suggested a good prognosis for EBV-positive OSCC patients as they discovered that no patients with EBV infection suffered from recurrence or death.

2.1.6.2. Fungal:

Oral candidiasis is an important opportunistic infection especially in immunocompromised patients like the human immunodeficiency virus (HIV) (Reichart 1999). Patients with oral epithelial dysplasia or OSCC had recorded a higher number of yeast in their oral cavity than those without (McCullough et al. 2002). The surfaces of oral cancers are often invaded by yeast with *Candida albicans* being the dominant species (Krogh 1990), Nagy et al. 1998).

2.1.6.3. Bacterial:

Syphilis infection has been associated with oral cancer especially on tongue (Binnie et al. 1983, Dickenson et al. 1995). Syphilitic-linked leukoplakia or carcinoma has been shown to occur predominantly on the dorsum of the anterior two-thirds of the tongue, which is an unusual cancer site (Binnie et al. 1983).

A study carried out between 1936-1968, reported that there was only 6.1% of the tongue carcinoma that were positive of syphilis (Meyer et al. 1970). In a study to explain the relationship of syphilis to cancer, showed that there was an increase in cancer incidence among people with syphilis though no conclusions may be reached concerning causality (Michalek 1994).

2.1.7. Others:

2.1.7.1. Diet:

Diets and nutrition have been indicated as very important factors in oral cancers. Researches with large sample sizes have uniformly shown that frequent consumption of vegetables, citrus fruits, fishes and vegetable oils, are the major features of low-risk diets for oral cancers adjusting for smoking and alcohol intake (Levi 1998, Franceschi 1999). Fruits and vegetables which are high in vitamin A, C, E, selenium and carotenoids have a protective effect in oral cancers, whereas meat and red chili powder are thought to be risk factors (Negri 2000, Johnson 2001, Zain 2001).

In a report by Negri et al. (2000), among seventeen selected micronutrients studied, protective effects were strongest for carotene, vitamin C, vitamin B6, folic acid, niacin and potassium. Indeed, lower level of several micronutrients such as vitamin B, folate, alpha and beta-carotene, lycopene and alpha tocopherol were found in the serum and buccal mucosa cells of chronic smokers (Gabriel et al. 2006).

The exact protective mechanism of these micronutrients is not clear now but it could be due to the antioxidative (Stahl et al. 2005) and suppression of cell proliferative abilities (Yoshida et al. 2005). These micronutrients showed their protective effect against cancers with their antioxidant activities, by reducing the free radical reactions that cause DNA mutation. They also modulate the metabolism of carcinogen in cells, which affect the transformation and differentiation of cell (Machlin 1987).

2.1.7.2. Occupation:

Occupation as a risk factor has been studied to a lesser extent. Epidemiological evidence exists for an association between workers exposed to formaldehyde and other manual workers such as printers, electronics workers, and textile workers had shown increased risk of oral cancers. (Vaughn TL 1986, Durbow 1984, Vagero 1983, Moulin 1986)

2.1.7.3. Immune defense:

Incidence of malignancy has been recorded to increase in chronic immunodeficiency states (Streilein 1991). OSSC has been reported in younger persons undergoing immunosuppressive regimes following organ transplantation (Varga 1991). However, the oral cancer incidence is stated to be very low with no evidence of particular preponderance in these patients (Thomas 1993).

2.1.7.4. Mouthwashes:

In 1979, Weaver and colleagues raised concerns regarding the use of mouthwash in increasing risk of OSCC. The main concern was the alcohol containing mouthwashes. Many researchers have studied the possibility of high content of alcohol in mouth rinses that might play a causative role in cancer. Several studies have reported cases of oral carcinoma in non-smokers and non-drinker who used alcoholic mouthwashes regularly for long period of time (Blot et al. 1988, Winn et al. 1991). It was found that alcohol concentration of 25% or greater had a greater risk of oral and pharyngeal cancer after adjusting alcohol drinking and tobacco use (Winn et al. 1991).

2.1.7.5. Maté:

The consumption of *Maté*, a tea-like beverage, has been suggested as a risk factor for oral cancer in South America region. However, the exact mechanism is still unknown (Goldenberg 2002).

2.1.7.6. Ethnicity:

Ethnicity strongly influence as a result of social and cultural practices, as well as influencing death rates owing to socioeconomic differences. Where cultural practices represent risk factors, their continuation by immigrants from high incidence regions to other parts of the world results in comparatively high cancer incidence in immigrant communities. For example among Indians living in Malay peninsula, the overall incidence of oral cancer has long been considerably higher than that among Malay or Chinese subjects. (Batsakis 2003)

2.2. Molecular Basis of Cancer:

2.2.1. Cell cycle and carcinogenesis:

There are internal and external regulators which control the progression of the cell cycle from its initial growth phase (G1) to its mitotic phase (M) that ultimately direct the fate of a cell either to form two daughter cells or to enter into resting state (G0). Deregulated cellular proliferation, arising from abnormal expression of genes that control cell cycle checkpoints (G1-S and G2-M phases), plays a critical role in tumorigenesis (Bartek et al. 1999).

Entry and progression of cells through the cell cycle are controlled by changes in the levels and activities of a family of proteins called cyclins. The levels of the various cyclins increase at specific stages of the cell cycle, after which they are rapidly degraded as the cell moves on through the cycle. Cyclin accomplish their regulatory functions by complexing with (and thereby activating) constitutively synthesized proteins called *cyclin-dependent kinases (CDK)* (Cordon-Cardo 1995). Different combinations of cyclins and CDKs are associated with each of the important transitions in the cell cycle, and they exert their effects by phosphorylating a late proteins, counter-regulatory proteins called *phosphatases dephosphorylate proteins)* (Murray 2004, Dongpo et al. 2006).

In normal circumstances, there is balance in the cell proliferation and cell death. However, in the event of carcinogenesis, the equilibrium is disturbed by three mechanisms: a) an increase in cell production rate, b) a reduced cell loss rate and c) simultaneous change in both rates (Wright & Alison 1984). The cell production rate and loss are governed by two large groups of genes: oncogenes and tumor suppressor genes.

2.2.2. Apoptosis (Cellular death):

Apoptosis refers to the most predominant form of physiological cell death that is used for the coordinated death of excess, hazardous or damage somatic cells. The central executors of this process are the caspases, a class of cysteine proteases that includes several representatives involved in apoptosis. These apoptotic caspases

undergo activating cleavage during apoptosis and between them; they cleave a range of substrate proteins to mediate the apoptotic process. These substrates are grouped according to their functions and two of them are the pro and anti- apoptotic proteins (Kerr 1971).

Currently, there are two recognized apoptotic pathways: Kerr et al. (1972).

1. The ancestral pathway: Release of cytochrome c from mitochondria, which formed complexes with two cytosolic proteins, the Apaf-1 and -3 which would in turn activate caspase-3 and the apoptotic cascade.
2. The death receptor pathway: This pathway involves the activation of specific group of transmembrane receptors of the tumor necrosis factor (TNF) receptor that initiates a signal transduction cascade, which leads to caspase-dependent programmed cell death.

2.3. Prognostic indicators in oral cancer:

2.3.1. Patient-related factors:

2.3.1.1. Age distribution of oral cancer

Oral cancer predominantly is a disease found in middle-aged and older persons (Neville 2002). The incidence of oral cancer increases with age in all parts of the world. The incidence of oral cancer at any age is comparatively low in western countries (2-6% of malignancies), but on the Indian sub-continent the rates were as high as 30-40% (Parkin et al. 1993).

However, in the past two to three decades, there has been an alarming increase in oral cancer especially among younger men in many Western countries (Johnson 2003) and Indian sub-continent (Gupta and Nandakumar 1999). In the West such as UK and France, 98% of oral and pharyngeal cases are in patients over 40 years of age. Studies from UK have reported rising trends in oral cancer particularly for tongue cancer among young adults (Johnson & Warankulasuriya1993).

In high-prevalence areas such as the Indian sub-continent, cases occur prior to the age of thirty-five due to heavy abuse of various forms of tobacco (Johnson 1991). Furthermore, a number of cases of oral cancer occur in both young and old patients often in the absence of traditional alcohol and tobacco risk factors and may pursue a particularly aggressive course Johnson (2001). In Sri Lanka, nearly 5% of oral cancer is diagnosed in young patients (Siriwardena et al. 2006).

Furthermore, a comprehensive literature review of risk factors for oral cancer in young people undertaken by Llewellyn et al. (2001) showed that most studies suggest that 4-6% of oral cancer now occur at ages younger than 40years. Information on many aspects of etiology for this disease in the young implicating occupational, familial risk, immune deficits and virus infections are meager. Besides, genetic instability has also been hypothesized as a likely cause (Llewellyn et al. 2001).

Clinicians from Tel Aviv University noted that oral tongue cancer was associated with worse 2-year disease-specific survival in patients younger than 45 years, leading them to conclude that oral tongue cancer appeared to follow a more aggressive course in younger individuals even though disease-specific survival at 5-years was similar (Popovtzer & Shpitzer, 2004).

2.3.1.2. Delayed diagnosis:

The delay in the diagnosis raises the probability of high tumor growth and spread, consequently worsens the prognosis (Allison et al. 1998). The patient with more hostile tumor develop symptoms earlier, seeking medical attention sooner, nevertheless, these patients still have to face a very serious effects, because these malignancies display a more aggressive biologic behavior (Massano et al. 2006). The failure to identify and diagnose premalignant and early cancerous oral lesions stems from several factors which include a lack of public awareness of signs, symptoms, and danger of oral cancer, insufficient awareness and training health care providers in oral cancer diagnosis and the inherent difficulty in distinguishing the sometimes subtle changes associated with early neoplastic changes from the more common benign and inflammatory lesion (Warnakulasuriya, et al. 1999, Rankin & Burznaski 1999).

2.3.2. Anatomical site:

The prevalence and incidence of oral cancer may differ between countries and is also dependent on the site of oral cancer. Different oral cancer sites may be associated with different lifestyle risk habits. Oral cancer in different sites may also have different behaviors leading to different prognosis.

2.3.2.1. Tongue cancer:

The tongue is the most common intraoral site for cancer, which has been shown in a number of studies (Moore et al. 2000). Nearly 75% of the oral carcinomas of the tongue arise in the anterior two thirds of the tongue, 20% occur on anterior lateral or ventral surfaces and only 4% occur on the dorsum (Neville & Day 2002, Murphy 2002).

The lateral borders and base of the tongue are the most common cancer areas and together with the floor of the mouth; represent the intraoral sites for cancer in many populations (Steward & Kleihues 2003). It has been suggested that the strong liking of these sites for intraoral cancer is due to the pooling of carcinogens in saliva in these food channels and reservoirs or "gutter zones" (Chen & Katz 1990, Johnson & Warnakalasuriya 1993). There are two possible reasons that carcinogens mixed with saliva constantly pool in these sites and these regions of the mouth are covered by thinner, non-keratinized mucosa, which provides less protection against carcinogens (Rumboldt & Day 2006).

Moore (2000) reviewed that the sites most at risk are tongue (ventral and lateral surfaces), floor of mouth, and anterior tonsillar and lingual aspect of the retromolar trigone. The typical carcinoma of the anterior two-thirds of the tongue presents as a painless, indurated ulcer on the lateral border. It is detected earlier than those of the posterior one-third and also tends to be better differentiated, and for this the posterior one-third is more aggressive with rapid invasion to the cervical nodes (Neville & Day 2002).

2.3.2.2. Buccal mucosa and lip cancer:

The vast majority of buccal mucosa cancers are located posteriorly. Usually the cancer extends into upper or lower sulcus (Pindborg & Reichart 1997). Carcinomas of the buccal mucosa can also be seen at the commissure or in the retromolar area. Most are ulcerated lumps and some arise from candidal leukoplakias. Cancer of the buccal mucosa is predominantly due to betel quid chewing habit, such as in India and Taiwan (Gupta & Nandakumar 1999, Lee et al. 2006).

Cancers of the lip usually arise in the vermillion border and the lower lip is most commonly affected. Cancers of the labial commissars are usually preceded by nodular leukoplakia, often associated with Candida infection (Batsakis 2003). Unlike intraoral cancers, cancers of the lip arise due to tissue changes caused by age and ultraviolet radiation, namely actinic or senile keratosis and elastosis (Silverman 2001, Steward & Kleihues 2003).

2.3.2.3. Floor of the mouth cancer:

Carcinoma of the floor of the mouth is commonly presented as painless inflamed superficial ulcer with poorly defined margins (Silvio et al. 2006) and is often located in the anterior part, either close to or in the midline. It represents 35% of all intra oral cancers and tends to increase in frequency among females (Pindborg & Reichard 1997, Neville & Day 2002).

The floor of the mouth is the second most common intraoral site for cancer in developed countries (Silverman 2001, Johnson 2001). It is ranked fourth despite distribution differs in developing countries (Gupta & Nandakumar 1999). Cancer of the floor of the mouth is more commonly associated with leukoplakia (Neville & Day 2002).

2.3.2.4. Gingiva and Palate cancer:

Carcinoma of the gingival and edentulous alveolar ridge may present as an ulceration and resemble inflammatory lesions. They are commonly associated with leukoplakia. Carcinomas of the alveolus or gingival mostly are seen in the mandibular premolar and molar regions, usually as a lump (epulis) or ulcer. The underlying alveolar bone is invaded in 50% of cases, even in the absence of radiographic changes, and adjacent teeth may be loose. There is a direct proportion between the incidence of gingival cancer and the usage of betel quid chewing among younger adults in Taiwan and India. (Lee et al. 2006, Gupta & Nandakumar 1999).

Palatal cancers are usually rare and are mostly seen in reverse smokers. Reverse smoking has been associated with a significant risk of malignant transformation due to the heat created by this habit, which usually develops as an ulcer lateral to midline of the hard palate (Neville & Day 2002, Gupta & Ray 2004, Pindborg & Reichart 1997). Reverse smoking is mostly found in some Southeast Asian, such as among the population in Philippine and India, and South American countries (Neville & Day 2002, Ortiz et al. 1996, Gupta & Ray 2004).

2.3.3. TNM staging of oral cancer

TNM system is a clinical staging system that deals with the anatomic extend of malignant solid tumors which is used for oral cancer. It allows the clinician to design treatment strategies, compare results and assess the likelihood of treatment success or determine the prognosis (Macluskey et al. 2004) and is established according to several criteria; tumor size, location, and extent (how far it has spread). Each letter in TNM has a specific meaning (T= the size of the primary Tumor, N = the status of the cervical lymph Nodes, M = the presence or absence cancer in sites other than the primary tumor [Metastasis]) (John, 2007).

However, the clinical TNM staging of the disease can be different from what is found after the excision and histopathological examination (pTNM) (Ogden & Macluskey 2000). Incidence of both false positive and false negative neck nodes is approximately 20% and fallibility of palpation metastatic neck disease is reportedly

more than 30% (Bryne et al. 1991). Cervical node metastasis may be classified into two categories: overt (clinical) or non-overt (occult) (Ferlito et al. 2003).

The more comprehensive and detailed the staging system, the more accurate and more predictive of prognosis the system becomes (Snehal & Jatin 2005). Mortality increases in relation to the stage at which the diagnosis of oral squamous cell carcinoma is made. Patients with stage III or IV lesions have a much poorer prognosis than those with stage I or II lesions (Oliver & John 1996).

2.3.4. Tumor Depth:

Tumor depth of invasion has been shown to be of major importance in predicting cervical metastasis (Fukano et al. 1997). Depth of invasion of >5mm had a significantly better prognosis than <5mm (Speight & Morgan 1993). This 5mm discerning point was also observed by Fukano (1997) where the incidence of cervical metastasis was increased markedly when the depth of invasion was over 5mm. Therefore, elective neck surgery should be performed on tumors with depth of invasion exceeding 5mm.

Tumor size and depth of invasion were highly correlated. A separate study by (Kristensen et al. 1999) indicated that patients with small tumors less than 2cm in diameter and larger tumors but a depth on invasion of less than 1cm were considered as a low risk group with a 5-years disease-free survival of 95%.

2.3.5. Histopathology grading of oral cancer:

There is no difference between squamous cell carcinoma of oral cavity and of the other sites at a microscopic level. In order to assess the tumor aggressiveness and hence prognosis of the patient, squamous cell carcinoma is graded based on the method described by Broders (1920). The grading is described by Pindborg & Reichart (1997) which is based on the degree of keratinisation, cellular and nuclear pleomorphism and mitotic activity.

Well and moderately differentiated tumors are to be grouped together as low grade and poorly differentiated and undifferentiated tumors as high grade. While a tumor shows different grades of differentiation, the higher grade determines the final categorization (Pindborg & Reichart 1997). In general, well-differentiated and moderately differentiated carcinomas (Grade 1 and 2) are seen more often than the poorly differentiated carcinomas (Grade 3) and undifferentiated carcinomas. Poorly differentiated carcinomas have a poor prognosis compared to well-differentiated and moderately differentiated carcinomas (Pindborg & Reichart 1997).

Several large studies during the seventies reported a correlation between histological grade and survival. Broders'/WHO grade alone recognized as a poor correlation with outcome and response to treatment in an individual patient (O-Charoenrat et al., 2003; Pindborg & Reichart 1997). The subjective nature of the assessment; small

biopsies from tumors showing histological heterogeneity and inadequate sampling; reliance on structural characteristics of the tumor cells rather than functional ones; and evaluation of tumor cells in isolation from the supporting stroma and host tissues have all been cited as possible explanations for the disappointing findings (Pindborg & Reichart 1997).

2.3.6. Tumor front:

The invasive edges of oral squamous cell carcinoma usually display different morphological and molecular characteristics than the more superficial parts of the tumor Bryne et al. 1995. Invasion may occur in the form of solid sheets, cords or islands of malignant cells and sometimes by dissociated individual cancer cells. The basement membrane may be more or less distinct, or completely absent.

Most molecular events occur at the tumor-host interface (invasive front) which are important for tumor spread such as gain and loss of adhesion molecules, secretion of proteolytic enzymes, increasing cell proliferation and initiation of angiogenesis Bryne 1998. Many mechanisms such as mechanism that control cell differentiation, migration, cell renewal or death (apoptosis) which are disturbed occur at the invasive front. Tumor at the invasive front usually shows a lower degree of differentiation and higher grade of cellular dissociation than the remaining areas of the tumor (Bryne 1998).

2.3.7. Molecular marker:

2.3.7.1. Oncogenes:

Oncogenes are mutated forms of genes that cause normal cells to grow out of control and become cancer cells. They are mutations of certain normal genes of the cell called proto-oncogenes. Proto-oncogenes are the genes that normally control how often a cell divides and the degree to which it differentiates. At the time when a proto-oncogene mutates into oncogenes, it becomes permanently activated and this inappropriate activation can involve mutation change into the protein leading to too quickly and uncontrolled division, which end by cancer (Ogden & Macluskey 2000).

Oncogenes are associated with different stages of neoplasia; some appear to be involved in tumor initiation and others in promotion, progression and metastasis (Fearon & Vogelstein 1990, Todd et al. 1997). Although oncogenes alone are not sufficient to transform a normal oral keratinocytes to a malignant one, they do appear to be important initiators to the process (Williams 2000, Todd et al. 1997).

Oncogenes are broadly represented by:

1) Growth factors or growth factor receptors (hst-1, int-2, EGFR/erbB, c-erbB-2/Her-2, sis)
2) Intracellular signal transducers (ras, raf, stat-3)
3) Transcription factors (myc, fos, jun, c-myb)

4) Regulators of cell-cycle (Cyclin D1)
5) Those involved in apoptosis process (bcl-2, Bax)

2.3.7.1.1. Cyclin D1:

Proto-oncogene that regulates cell cycle; its product, CCND1, phosphorylate *Rb*, promoting the transition G1→S. Cyclin D1 activity is inhibited by several tumor-suppressor genes. The amplification and overexpression of this gene are independent prognosis factors in several tumors, including head and neck squamous cell carcinoma (Meyer et al. 2002, Miyamoto et al. 2003 and Schneeberger et al. 1998). Increased expression of cyclin D1 is associated with the presence of regional nodal metastases, and advanced tumor stage. Therefore, it may be a useful prognostic indicator. (Scully et al. 2000a)

2.3.7.2. Proto-Oncogene:

Proto-oncogenes are genes present in normal cells that determine cell growth, proliferation and differentiation. It is capable of regulating growth by producing various protein products that form intracellular communication network, which controls cell growth and when altered by mutation, becomes an oncogene that can contribute to cancer [Fearon & Vogelstein 1990]. The protein products of proto-oncogenes control growth at one or more steps in the growth-signaling pathway. Some proto-oncogenes products are peptides that stimulate cell proliferation (growth factors) or cell receptor proteins for growth factors (growth factor receptors). Some are protein involves in the transduction of signals within cells (intracellular signal-

transuding proteins) and some can regulate the production of messenger RNA (mRNA) from genes (nuclear transcription factors) (Todd et al. 1997).

2.3.7.3. Tumor-suppressor genes:

Tumor suppressor genes are normal genes that slow down cell division, repair DNA mistakes, and tell cells when to die (a process known as apoptosis or programmed cell death). When tumor suppressor genes do not work properly, cells can grow out of control, which can lead to uncontrolled cell growth and lead to cancer (Ayo-Yusuf OA et al. 2004). With further advancement in techniques in somatic cell genetics, series of experiments proved the following:

- A set of genes exists that function in a dominant fashion to block the tumorigenic potential of cancer cells.
- The cancerous cells must be sustaining mutations in both alleles of these genes to gain the ability to produce tumors in the host or transplanted animals (Murphy 2002).

2.4. Survival rate for oral cancer patients:

Intra-oral cancer is particularly lethal, that of the lip less so, the crude five years survival rates being 30-40% and 90% respectively (Johnson 1999). According to Mashberg (2000), survival rates for cancers of the oral cavity and oropharynx have remained constant during the last 20-30 years at approximately 40-50%.

There have been great advances made in the management of oral cancer, from improved diagnostic imaging of the tumor to sophisticated reconstructive procedures including oral implantology to restore the dentition (Hollows et al. 2000). Due to the improvement in the technology and surgical techniques, the survival rate itself improved in recent decades (Johnson 1999, Hollows et al. 2000).

Some studies show that the prognosis for survival depends on the stage of the disease at the time of diagnosis (Israel 1986). Most of the oral and oropharyngeal cancers at the time of the diagnosis are symptomatic late stage disease (stage III or stage IV) with at least 50% revealing regional cervical metastasis (Mashberg et al. 2000, Rumboldt & Day 2006).

2.5. Techniques of Identification of Molecular marker:

Gene alteration in OSCC has been previously investigated by different techniques to explore their role in the carcinogenesis and progression of this neoplasia. These techniques include:

1) Immunohistochemistry.
2) DNA content analysis.
3) Laser captures microdissection (LCM).
4) Proteomics.
5) Molecular genetics:
   a) FISH technique is employed to detect the chromosome changes directly.
   b) Array Technologies
   c) Southern Blot Hybridization.
   d) Polymerase Chain Reaction (PCR)

Molecular base methods of cancer diagnosis can be applied for different purposes in the evaluation of cells and tissues. The most important purpose of diagnosis is to distinguish neoplastic from reactive processes and malignant from benign neoplasm beside to establish the likely tissue of origin by assessing the features of tissue differentiation displayed by the tumor.

With the introduction of new diagnostic methods and variable approaches to diagnosis developed during the past few years, it is clear that critical diagnostic pathways need to be elaborated and evaluated to provide guidance in test use. For example, in some cases, a straightforward light microscopic examination of a smear of exfoliated cells may suffice for diagnosis and therapy; in other cases, application of recently developed molecular genetics techniques is necessary to establish the nature of the lesion and guide therapy.

2.5.1. Immunohistochemistry:

Immunohistochemistry is an important tool for dissecting multiple cell populations in non homogeneous tumoral tissues. Detection of antigens specific for each two or more cell types within the same lesion can define tumors showing diversion of different cell lines. Immunohistochemistry can also identify the reactive cells that infiltrate the tumor from malignant cells, when tumor cells are difficult to differentiate from reactive elements by routine histochemical stains alone (Zanardi et al. 2007)

2.5.2. FISH technique:

Fluorescent In Situ hybridization (FISH) technique is a study of cytogenetic changes in solid tumors by in situ hybridization using chromosomes specific DNA probes. DNA sequences can be detected in interphase nuclei (interphase cytogenetic). Recently, a number of FISH technique variants used to detect chromosome or genomic imbalances in interphase cells have flourished (Hackel & Varella-Garcia M 1997). Essentially, FISH allows for a comprehensive characterization of the chromosomal alterations and assessment of topographic distribution of the most prominent changes in tumor on a single cell basis, yielding information on tumor heterogeneity and progression. In addition, FISH technique is fast to perform and only requires a small amount of cells, which make it more suitable for routine screening of tumorigenesis (El-Naggar et al.1996, Hemmer & Prinz 1997 and Barrera et al. 1998).

FISH technique overcome many of the practical problems with conventional cytogenetics by permitting more specific staining of any given region of the genome. In this technique, DNA probes derived from the regions of the genome under investigation are hybridized to metaphase chromosomes deposited on microscope slides or to chromatin within intact interphase cells. Hybridization to chromosomes is monitored by fluorescence, usually by an indirect method using probes that have been synthesized with modified nucleotides tagged with biotin or digoxigenin.

The hybridized probe is recognized by antibodies directed against molecular tag coupled to a fluorochrome. Direct tagging of DNA probes with fluorescent molecules is also possible. The results of hybridization are examined under a standard fluorescence microscope or one fitted with a digital camera that transmits the image to a computer for processing of the signals (Hyunmin et al. 2005).

Presently, most of the probes used contain tens to hundreds of kilo bases of DNA and these can be propagated in bacteriophage and cosmid cloning vectors or as yeast or bacterial artificial chromosomes. FISH as a method to detect chromosomal abnormalities in cell has many advantages. Hybridization produces a more reproducible signal that makes interpretation of the results easier. Additionally, the hybridization signal is more specific than conventional banding. The most important advantage is FISH technique on interphase cell is very fast, simple and robust which take hours rather than days for routine cytogenetic. It also avoids the expense and pitfalls of culturing cells. The technique can also be carried out on formalin-fixed, paraffin-embedded tissues (Francesco et al. 2008).

Disadvantage of FISH relative to conventional cytogenetic is that the only region analyzed is corresponding to the probe. However, the concurrent use of many differently tagged fluorescent probes in combination can decorate numerous regions of metaphase chromosomes in various colors and produce bands almost comparable

in number to conventional cytogenetic but with greater specificity for individual regions (Lengauer et al. 1993).

Initially FISH technology focused on research field, but soon was applied to clinical use and has proved sufficiently sensitive and reliable to narrow the gap between classical karyotyping and highly sensitive molecular techniques. (Van Dekken 1990, Giwereman et al. 1990, Nederlof & Robinson 1989, Hopman et al.1989, Emmerich et al.1989).

The number of FISH signals, which was found to be constant during the cell cycle, in the interphase nucleus and in the condensed chromosomes indicates the chromosome copy number independent from the cell cycle stage (Cremar 1988, Hopman et al.1988 and Hopman et al.1989).

Limitations to the FISH assay include the technical artifacts that leads to signals loss or gain. For instance, target sequence may remain undetected due to counterstain that obscures small or weak hybridization signals, high stringency of post hybridization washes or lack of probe penetration into the nucleus. Conversely, cells in the G2 or late S phase with decondensed DNA may display significantly separated signals for the sister chromatids, leading to an incorrect interpretation as hyper diploid (Eastmond et al.1995). In addition, the centromeric sequences are highly repetitive sequences in the genome and less specific homology may be recognized as cross-hybridization. Two factors must be considered in the selection of an optimal set of FISH probes for tumor screening. Firstly, the probes should have high hybridization

efficiency. Secondly, they are expected to exhibit a high sensitivity in detecting aneuploidy.

The detection specificity of individual chromosomes is mainly determined by the stringency condition under which the DNA probes are hybridized. A high percentage form amide (60%) in the hybridization and washing buffers for all chromosome probes are applied to avoid interaction with minor binding sites.

The FISH sensitivity using chromosome-specific DNA probes nuclei is mainly dictated by the treatment prior to FISH. Protease treatment, which removes a large part of the nuclear protein, will result in 90-98% evaluable cases and in a low percentage of false-negative chromosomes aneuploidy detections.

CHAPTER THREE

MATERIALS and METHODS

This is a preliminary study which is part of a major project on “Oral Cancer and Precancer In Malaysia- Risk Factors, Prognostic Markers, Genetic Expression and Impact On Quality of Life”. MEC ethics approval number is **DF OS0905/0017(p) at 15th of May 2009.**

Additional ethnics approval for this part of project was obtained from the ethics committee, Faculty of Dentistry, University Of Malaya.

3.1. Materials:

This study, is based on 50 OSCC paraffin embedded tissue samples of surgical specimens (resected tumors) available in the archives of the Oral Pathology and Oral Medicine department and Oral Cancer Research Coordinating Center (OCRCC) from the year 2004 to 2010. All the tissues were previously fixed in 10% buffered formalin and processed with standard histological embedding techniques. Power and sample size calculation software V2.1.31 was used to obtain a minimal size sample of 45. However, we round up the number to 50 in order to have equal number of samples for tongue and B.M. SCC.

3.1.1. Inclusion criteria:

- Archival tissue from primary squamous cell carcinoma (OSCC) of the tongue and buccal mucosa taken in this study which were never exposed to radiotherapy or chemotherapy treatment previously.
- Cases of histopathologically confirmed SCC of tongue and buccal mucosa from 1st January 2004 to 1st June 2010.

3.1.2. Exclusion criteria:

- Samples from patients who have been exposed to radio/chemotherapy.
- Samples from recurrent oral squamous cell carcinoma despite the sites mentioned previously.

3.1.3. Clinicopathological data:

Data on demography, tumor site, histopathological grade, pattern of invasion, tumor depth, pathological TNM staging and survival were obtained from the Malaysian Oral Cancer Tumor and Database (MOCTDBS) and reevaluation of the histopathological data were done by the author and the second supervisor (an experienced pathologist) to increase the reliability in the present study.

3.1.3.1. Modified Broder's malignancy grading system:

This grading system was used to evaluate the histopathogical grading. It subjectively assesses the degree of differentiation and keratinization of tumor cells (Pindborg & Reichart 1997). There are three grades in this grading system:

Grade 1: Well-differentiated: Histological and cytological features closely resemble those of squamous epithelial lining of the oral mucosa. There are varying proportions of basal and squamous cells with intercellular bridges; keratinization is a prominent feature; few mitotic figures are seen and atypical mitosis or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal.

Grade 2: Moderately differentiated: This is a neoplasm with features intermediate between well and poorly differentiated. Compared with well-differentiated squamous cell carcinomas, these have less keratinization and more nuclear and cellular pleomorphism; there are more mitotic figures and some are abnormal in form; intercellular bridges are less conspicuous.

Grade 3: Poorly differentiated: Histologically and cytological there is only a slight resemblance to the normal stratified squamous epithelium of the oral mucosa. Keratinization is rarely present and intercellular bridges are extremely scarce; mitotic activity is frequent and atypical mitoses can readily be found; cellular and nuclear pleomorphism are obvious and multinucleated cells may be frequent.

3.1.3.2. Pattern of invasion:

According to the Royal College of Pathologists (Helliwell & Woolgar 2000), the cut-off for prognostic purposes appears to be cohesive (pattern 1 and 2) and non-cohesive (pattern 3 and 4).

3.1.3.3. TNM staging:

Data of the pathological description of the tumors (tumor size, nodal status and distant metastasis) were derived from HPE report. The TNM staging was previously charted by the oral pathologist based on the International Union Against Cancer (Appendix 3).

3.2. Methods

3.2.1. Probe of FISH technique:

SO LSI Cyclin D1 DNA probe (Vysis, Inc. Downers Grove, IL, USA) which hybridizes to band 11q13 of human chromosome 11, was used. The centromeric probe for chromosome 11 (alpha satellite) was used for dual color FISH.

3.2.2. Analysis of chromosome copy number:

The pathologist selected areas for analysis by comparing hybridized slides to a corresponding H&E stained section. The hybridized signals appear as small spot since the region of a chromosome occupies only a small region of the nucleus. At least 200 nuclei were scored using a 100X objective in each defined histological area, and each nucleus was assessed for the chromosome copy number. If the signals ratio of the orange signals to the green signals is 2 or more than 2 then it is considered positively amplified.

Scoring criteria as follows:

1. Cytoplasmic materials should not cover the nuclei.
2. No overlapping observed between the nuclei.

3. Minor hybridized spots, which can be recognized as smaller size and lower intensity was excluded.
4. Signals can be counted on well separated signal spot.

3.2.3. Specimen processing:

Three µm thick sections were incubated at 37°C overnight and deparaffinized by washing in xylene rehydrated in graded ethanol and distilled water. After incubation in 0.2 M HCl at room temperature for 20 minutes, they were heat-pretreated in citrate buffer (2 × SSC, pH 7.0) at 80°C for 30 minutes. They were then digested with protease buffer at 37°C for 80 minutes, rinsed in 2 × SSC at room temperature for 3 minutes and dehydrated in graded ethanol (70, 85, and 100%) for 2 minutes each. For each slide, 1-µl of probe was mixed with 2-µl purified H2O and 7µl LSI hybridization buffer and applied to the dry slide, the tissue area was coverslipped and sealed with rubber cement. The slides were then incubated in a moist chamber (Hybridizer Instrument for in situ hybridization, DAKO, S2450, Denmark) for denaturation at 82°C for 5 minutes and hybridization at 37°C for about 16 hours.

The following day post hybridization washes were performed in 0.4 × SSC/0.3% NP -40 at 73°C for 2 minutes to remove non-specifically bound probe and in 2 × SSC/0.1% NP-40 at room temperature for 2 minutes and after application of 5 µL of mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI), the tissue area was coverslipped. These slides were viewed under a fluorescence microscope (BX 16, OLYMPUS, Tokyo, Japan).

3.2.4. Principle:

Target DNA, after fixation, is treated with heat to denature the double-stranded DNA, rending it single-stranded. The target DNA is thus available for annealing to a similarly denatures, single-stranded, fluorescently labeled DNA probe which has completely sequence. Following hybridization, unbound and non-specifically bound DNA probe is removed by s series of stringent washes and the DNA counterstained for visualization. Fluorescent microscopy then follows the visualization of the hybridized probe on the target material.

3.3. Statistical analysis

Categorical data was statistically analyzed by Chi-square and Fisher's exact test. Survival curves were constructed using the Kaplan-Meier method and compared using the log rank test. The level of significance was set at *p* less than 0.05. All statistical analyses were performed using SPSS 18 software (SPSS, Chicago, IL).

3.4. Expected output:

The finding may indicate the possibility of Cyclin D1 as a prognostic marker and therefore may predict the outcome of the patients treated for OSCC.

CHAPTER 4

RESULTS

4.1. Introduction

This cross-sectional study is based on fifty OSCC paraffin embedded tissue samples of surgical specimens obtained from the archives of the Department of Oral Pathology, Oral Medicine and Periodontology and the Oral Pathology Diagnostic Laboratory. Equal number of tongue and buccal mucosa OSCC were included. The related data was obtained from the Malaysian Oral Cancer Database and Tumor Bank System (MOCDTBS) coordinated by the Oral Cancer Research Coordinating Center (OCRCC). PASW 18.0 software was used to conduct the descriptive, survival and inferential statistical analysis.

4.1.1. FISH technique evaluation criteria:

Evaluation of the preparation was performed by counting at least 200 nuclei per slide, according to criteria described by Hopman et al. 1988.

4.1.2. Image analysis:

Enumeration of the florescent signals was performed in at least 200 nuclei per slide under objective power of 100X, using an Olympus florescent microscope BX61 equipped with single band sets for DAPI, Fluorescein Isothiocyanate (FITC) and spectrum Orange to discriminate the color signals of green for chromosome 11 centromeric DNA and orange for *CCND1* during scoring. Images for documentation were then captured using a spectral imaging camera and processed case data manager expo 5.0. (Figure 4.1)

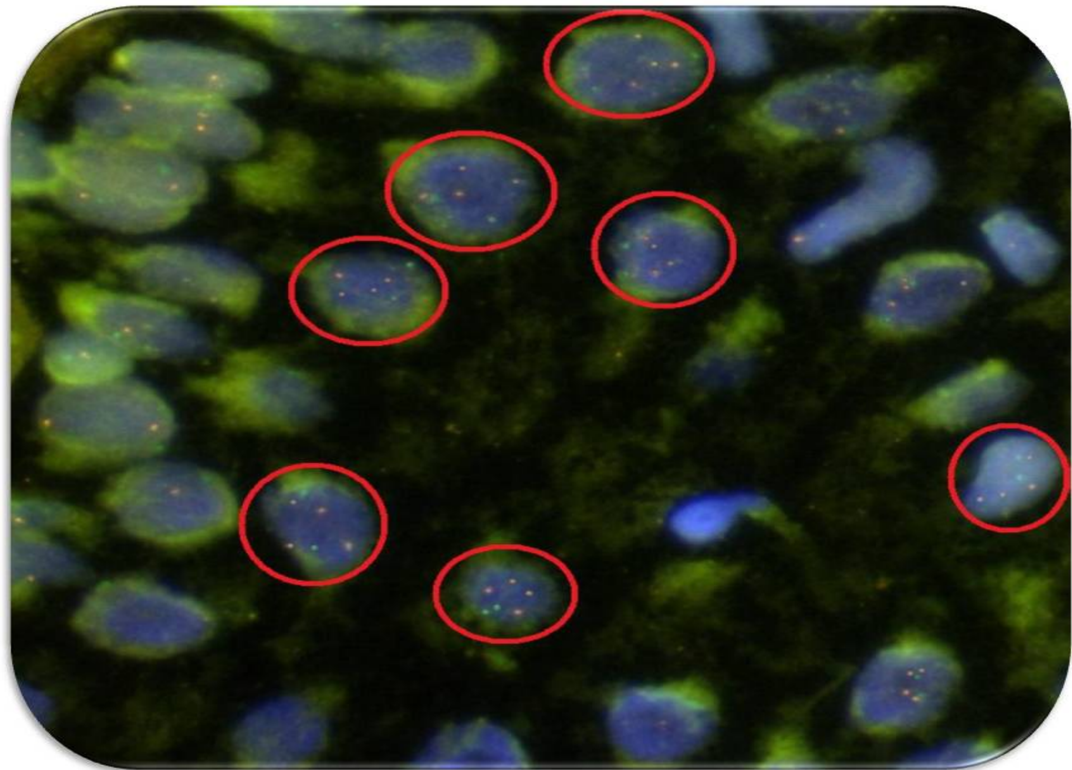

Figure 4.1 FISH staining showing green (chromosome 11) and orange (Cyclin D1) signals and amplification ratio in nuclei marked by the red circles.

4.2. Sociodemographic characteristic

This study was conducted on samples from19 males (38%) and 31 females (62%). The table below provide the total number of patients with oral squamous cell carcinoma according to gender, where we can find the majority of the patients were Indians followed by Malay then Chinese. Indian females are the largest group in this study. (Table 4.1)

The age of the patients ranged from 26-94 years with mean of 60 years old (Table 4.2). Majority of patients (56%) were more than or equals to 60 years old.

Table 4.1 Demographic distribution according to gender and ethnicity

Gender/ Ethnicity	I n (%)	M n (%)	C n (%)	Total n (%)
Male	10 (29.4%)	4 (40%)	5 (83.3%)	19 (38%)
Female	24 (70.6%)	6 (60%)	1 (16.7%)	31 (62%)
Total	34 (68%)	10 (20%)	6 (12%)	50 (100%)

I=Indian M=Malay C=Chinese

n=Number

Table 4.2 Distribution of cases according to age

Demographic parameters			
Age (Years)	Tongue n (%)	Buccal mucosa n (%)	Total n (%)
<60 yrs	9(36%)	13(52%)	22 (100%)
≥60 yrs	16(64%)	12(48%)	28 (100%)

4.3. Clinicopathological features

4.3.1. Tumor site:

The current study include half the number of total cases (n=50) from tongue SCC 25 (50%) and the other half from buccal mucosa SCC 25(50%). (Table 4.3)

4.3.2. Broder's classification

Twenty cases (40%) were graded as well-differentiated OSCC in this study. Moderately differentiated OSCC was seen in 26(52%) and poorly differentiated OSCC in 4 (8%). (Table 4.3)

4.3.3 Pattern of invasion

In this study majority of patients 44(88%) had non-cohesive type (type 3+4) pattern of invasion. Only six (12%) of cases had cohesive type (type 1+2) invasion of the tumor front. (Table 4.3)

4.3.4. Tumor size (pT)

In this study, histopathological features such as greatest tumor dimension (T), lymph node metastasis (N) and pathological TNM staging (pTNM) were studied. More than half (56%) had greatest tumor dimension more than 2 cm but not more than 4 cm in greatest dimension. Five cases had tumor size of 2 cm or less in greatest dimension, where as 13 samples were more than 4 cm in greatest dimension. Four cases had the tumor size more than 4 cm with invasion into adjacent structures. (Table 4.3)

4.3.5. Lymph node metastasis

Twenty seven samples in the study showed positive lymph node metastasis.

4.3.6. pTNM stage

In the current study, the pathological TNM staging was grouped into early stage (I+II) which represented 36% and advanced stage (III+IV) which presented the majority of cases 64%. (Table 4.3)

4.3.7 Tumor depth

In addition to pTNM staging, the tumor depth was also evaluated. Almost all cases had tumor depth of less than 5 mm or more. (Table 4.3)

Table 4.3 Clinicopathological features

Parameters	Tumor Site		
Histopathological grade	**Tongue n (%)**	**Buccal Mucosa n (%)**	**Total n (%)**
Well	8(32%)	12(48%)	20 (40%)
Moderately	13(52%)	13(52%)	26 (52%)
Poorly	4(16%)	0(0%)	4 (8%)
Pattern of invasion			
Cohesive	1(4%)	5(20%)	6 (12%)
Non-cohesive	24(96%)	20(80%)	44 (88%)
Tumor size (pT)			
pT≤ 2 cm	3(12%)	2(8%)	5 (10%)
2 < pT ≤ 4cm	12(48%)	16(64%)	28 (56%)
pT > 4cm	9(36%)	4(16%)	13 (26%)
Any pT with invasion	1(4%)	3(12%)	4 (8%)
lymph node metastasis(pN)			
N0	14(56%)	9(36%)	23 (46%)
N+ve	11(44%)	16(64%)	27 (54%
pTNM stage			
Stage I + II (early)	10(40%)	8(32%)	18 (36%)
Stage III + IV (advanced)	15(60%)	17(68%)	32 (64%)
Tumor Depth			
< 5 mm	2(8%)	1(4%)	3 (6%)
≥ 5 mm	23(92%)	24(96%)	47 (94%)
Total	25(50%)	25(50%)	50 (100%)

N0= no regional lymph node metastasis

N+ve= Positive regional lymph node metastasis

4.4. Cyclin D1 amplification:

Cyclin D1 amplification was positive in 36 out of 50 cases and more than half of the positive samples were tongue SCC. (Table 4.4)

Table 4.4 cyclin D1 amplification

Cyclin D1 amplification	Tongue	Buccal Mucosa	Total n (%)
Positive	22(88%)	14(56%)	36 (72%)
Negative	3(12%)	11(44%)	14 (28%)
Total	25(50%)	25(50%)	50 (100%)

4.4.1. Association between sociodemographic characteristic and cyclin D1 amplification:

4.4.1.1. Association between age and cyclin D1 amplification based on tumor site:

Fisher Exact test of independence was used to determine if cyclin D1 amplification is dependent on age. Given α = 0.05, the results suggest lack of dependency. No significant difference between age and cyclin D1 amplification in tongue and buccal mucosa SCC (tongue *p* value=0.287, buccal mucosa *p* value= 0.163) was noted. (Table 4.5)

Table 4.5 Association between age and cyclin D1 amplification

Tumor Site	**cyclin D1 amplification**			
	Age	**Positive n (%)**	**Negative n (%)**	***p*-value**
Tongue	< 60yrs	7(31.8%)	2(66.7%)	0.287**
	≥ 60yrs	15(68.2%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	< 60yrs	9(64.3%)	4(36.4%)	0.163*
	≥ 60yrs	5(35.7%)	7(63.6%)	
Total n (%)		14(100%)	11(100%)	

*Chi-square test was used.

**Fisher exact test was used.

4.4.1.2. Association between gender and cyclin D1 amplification based on tumor site:

A 2X2 Fisher Exact test of independence was used to determine if cyclin D1 amplification is dependent on gender for each tumor sites. Given $\alpha = 0.05$, the results suggest lack of dependency, tongue *p*-value= 0.469 and buccal mucosa *p*-value=0.102 (Table 4.6). Although amplification of cyclic D1 is more significantly by demonstrated in tongue tumor, gender has no influence on the amplification of cyclin D1.

Table 4.6 Association between gender and cyclin D1 amplification based on tumor site

Tumor Site	cyclin D1 amplification			
	Gender	**Positive n (%)**	**Negative n (%)**	***p*-value**
Tongue	Male	10(45.5%)	2(66.7%)	0.469*
	Female	12(54.5%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	Male	2(14.3%)	5(45.5%)	0.102*
	Female	12(85.7%)	6(54.5%)	
Total n (%)		14(100%)	11(100%)	

*Fisher exact test was used.

4.4.1.3. Association between ethnicity and cyclin D1 amplification based on tumor site

The results suggest an insignificant association between ethnicity and cyclin D1 amplification in tongue and buccal mucosa (tongue *p*-value=0.830, buccal mucosa *p*-value=0.072). (Table 4.7)

Table 4.7 Association between ethnicity and cyclin D1 amplification based on tumor site

Tumor Site	**cyclin D1 amplification**			
	Ethnicity	**Positive n (%)**	**Negative n (%)**	***p*-value**
Tongue	Malay	6(27.3%)	1(33.3%)	0.830*
	Indian	11(50%)	1(33.3%)	
	Chinese	5(22.7%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	Malay	0(0.0%)	3(27.3%)	0.072**
	Indian	14(100%)	8(72.7%)	
	Chinese	-	-	
Total n (%)		14(100%)	11(100%)	

*Chi-square test was used.

**Fisher exact was used.

4.4.2. Association between clinicopathological features and cyclin D1 amplification:

4.4.2.1. Association between Tumor site and Cyclin D1 amplification (Tongue and Buccal Mucosa SCC)

A 2X2 chi-square test of independence was used to determine if cyclin D1 amplification is dependent on tumor site. Given α = 0.05, the results suggest dependency, χ2 (one, N = 50) = 6.349. A significant result was observed between cyclin D1 amplification and SCC of tongue and buccal mucosa with cyclin D1 amplification positive in 22 of tongue SCC cases whereas only in 14 of buccal mucosa SCC cases were positive. (*p*-value=0.012). (Table 4.8)

Table 4.8 Association between the Amplification of Cyclin D1 and Tumor Sites (Buccal Mucosa and Tongue SCC)

Tumor Site	Cyclin D1 amplification				
	Positive n (%)	**Negative** n (%)	**Total**	χ^2	**P-value**
Tongue	22 (88%)	3 (12%)	25(100%)	6.349	0.012*
Buccal Mucosa	14 (56%)	11 (44%)	25(100%)		

*Chi-square test was used.

4.4.2.2. Association between modified Broder's grading and cyclin D1 amplification based on tumor site:

No statistical significant result was observed between cyclin D1 amplification and modified Broder's grading in tongue and buccal mucosa SCC. (Tongue *p*-value=0.157, buccal mucosa *p*-value=0.163). (Table 4.9)

Table 4.9 Association between modified Broder's grading and cyclin D1 amplification based on tumor site

Tumor Site	**cyclin D1 amplification**			
	Modified Broder's grading	**Positive n (%)**	**Negative n (%)**	***p*-value**
Tongue	Well	6(75%)	2(25%)	0.157*
	Moderately	12(92.3%)	1(7.7%)	
	Poorly	4(100%)	0(0.0%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	Well	5(41.7%)	7(58.3%)	0.163*
	Moderately	9(69.2%)	4(30.8%)	
	Poorly	-	-	
Total n (%)		14(100%)	11(100%)	

*Chi-square test was used.

4.4.2.3. Association between pattern of invasion and cyclin D1 amplification based on tumor site:

In this analysis, the association between cyclin D1 amplification and pattern of invasion was performed based on tumor sites (Tongue and Buccal mucosa). No significant result was observed between cyclin D1 amplification and the pattern of invasion at the invasive front in both tongue and buccal mucosa SCC. (Table 4.10)

Table 4.10 Association between pattern of invasion and cyclin D1 amplification based on tumor site

Tumor Site	**cyclin D1 amplification**			***p*-value**
	Pattern of invasion	**Positive n (%)**	**Negative n (%)**	
Tongue	COHESIVE	1(4.5%)	0(0.0%)	0.880*
	NON-COHESIVE	21(95.5%)	3(100%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	COHESIVE	1(7.1%)	4(36.4%)	0.096*
	NON-COHESIVE	13(92.9%)	7(63.6%)	
Total n (%)		14(100%)	11(100%)	

*Fisher-exact test was used.

4.4.2.4. Association between tumor greatest dimension (pT) and cyclin D1 amplification:

In this analysis, the association between cyclin D1 amplification and tumor greatest dimension (pT) was studied in both tongue and buccal mucosa. It is found that tumor more than 4cm in greatest dimension of tumor with invasion demonstrate cyclin D1 amplification.

However, in this study only tongue SCC cases shown a statistically significant association between of tumor invasion with positive amplification of cyclin D1. (Tongue p-value=0.019, Buccal mucosa p-value=0.267). (Table 4.11)

Table 4.11 Association between tumor greatest dimension (pT) and cyclin D1 amplification

Tumor Site		cyclin D1 amplification		
	pT	Positive n (%)	Negative n (%)	*p*-value
Tongue	pT≤ 2 cm	1(4.5%)	2(66.7%)	0.019*
	2 < pT ≤ 4cm	11(50%)	1(33.3%)	
	pT > 4cm	9(40.9%)	0(0.0%)	
	Any pT with invasion	1(4.5%)	0(0.0%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	pT≤ 2 cm	1(7.2%)	1(9.1%)	0.267*
	2 < pT ≤ 4cm	7(50%)	9(81.8%)	
	pT > 4cm	3(21.4%)	1(9.1%)	
	Any pT with invasion	3(21.4%)	0(0.0%)	
Total n (%)		14(100%)	11(100%)	

*Chi-square was used.

4.4.2.5. Association between lymph node metastasis (pN) and cyclin D1 amplification

Twenty-seven cases had regional lymph node metastasis, out of which 21 cases demonstrated positive cyclin D1 amplification. However, 15 cases without regional lymph node metastasis also showed cyclin D1 amplification. Therefore, no statistically significant result was observed. (Table 4.12)

Table 4.12 Association between lymph node metastasis (pN) and cyclin D1 amplification

Tumor Site		cyclin D1 amplification		*p*-value
	pN	**Positive n (%)**	**Negative n (%)**	
Tongue	N0	12(54.5%)	2(66.7%)	0.593*
	N+ve	10(45.5%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	N0	3(21.4%)	6(54.5%)	0.098*
	N+ve	11(78.6%)	5(45.5%)	
Total n (%)		14(100%)	11(100%)	

*Chi-square test was used

4.4.2.6 Association between pathological TNM staging (pTNM) and cyclin D1 amplification based on tumor site

The association between cyclin D1 amplification and pathological TNM staging (pTNM) (Stage I + II and Stage III + IV) was insignificant for both tumor sites. The test was used to analyze the data (α = 0.05). (Table 4.13)

Table 4.13 Association between pathological TNM staging (pTNM) and cyclin D1 amplification based on tumor site

Tumor Site	cyclin D1 amplification			
	pTNM	**Positive n (%)**	**Negative n (%)**	***p*-value**
Tongue	Stage I + II(early)	8(36.4%)	2(66.7%)	0.346*
	Stage III + IV (advanced)	14(63.3%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	Stage I + II(early)	3(21.4%)	5(45.5%)	0.199*
	Stage III + IV (advanced)	11(78.6%)	6(54.5 %)	
Total n (%)		14(100%)	11(100%)	

*Fisher exact test was used.

4.4.2.7. Association between tumor depth and cyclin D1 amplification based on tumor site

In this study the association between the cyclin D1 amplification and tumor depth (less than 5 mm and more than or equal to 5 mm) was studied for both tongue and buccal mucosa SCC. A significant result was observed between cyclin D1 amplification and tumor depth in tongue SCC whereas no significant was observed in buccal mucosa SCC, (Tongue *p*-value=0.010, buccal mucosa *p*-value=0.440) (Table 4.14).

Table 4.14 Association between tumor depth and cyclin D1 amplification based on tumor site

Tumor Site	cyclin D1 amplification			*p*-value
	Tumor depth	Positive n (%)	Negative n (%)	
Tongue	< 5 mm	0(0.0%)	2(66.7%)	0.010*
	≥ 5 mm	22(100%)	1(33.3%)	
Total n (%)		22(100%)	3(100%)	
Buccal Mucosa	< 5 mm	0(0.0%)	1(9.1%)	0.440*
	≥ 5 mm	14(100%)	10(90.9%)	
Total n (%)		14(100%)	11(100%)	

***Fisher exact test was used.**

4.5.1. Kaplan-Meier survival analysis (KMSA)

The survival rate was 47.2% for cyclin D1 amplification positive patients and 57.1% for negative patients(Fig 4.1). According to Log Rank (Mantel-Cox) test, the survival rate is significantly different (p=0.009) between positive and negative patients. (Table 4.15)

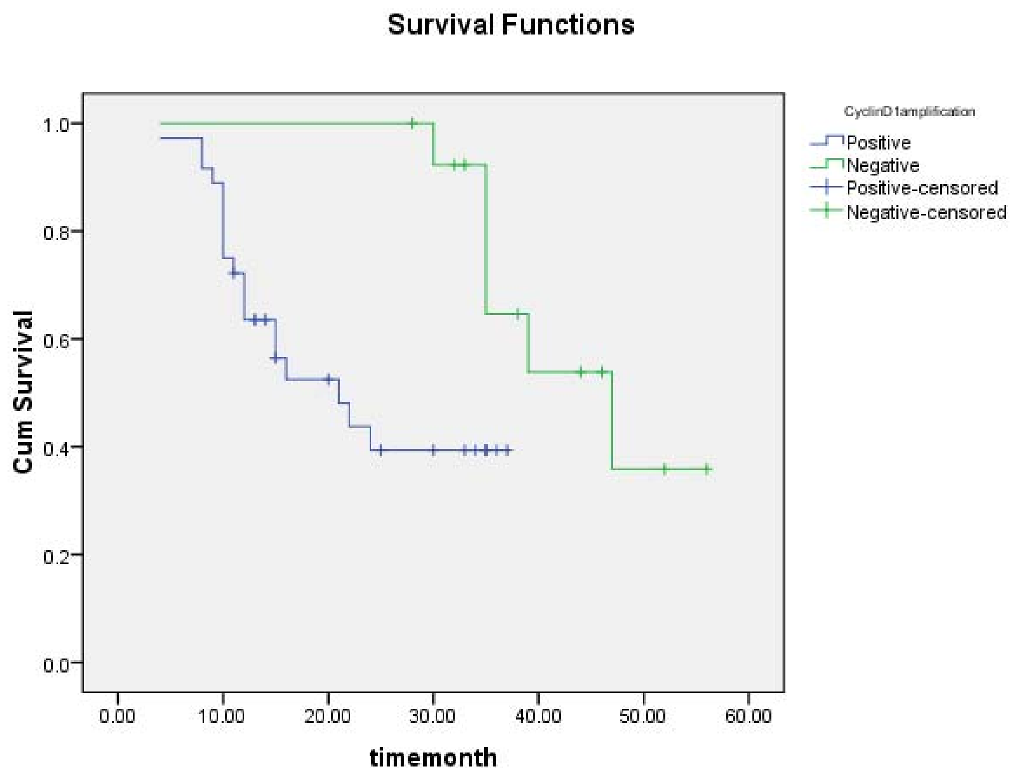

Figure 4.2 Survival rate for the sample (n=50) in relation with Cyclin D1.

Table 4.15 Log rank test to compare between survival rates between positive and negative cyclin D1 amplification.

Log Rank (Mantel-Cox)	Chi-Square	Df	*p*-value
Over all Sample	15.938	1	$p<0.001$

4.5.2. Kaplan-Meier survival analysis (KMSA) based on tumor site

The survival rate was 60% for tongue SCCand 40% for buccal mucosa SCC. According to Log Rank (Mantel-Cox) test, the survival rate is not significantly different (*p*-value=0.408) between tongue and buccal mucosa SCC patients. (Fig 4.2)

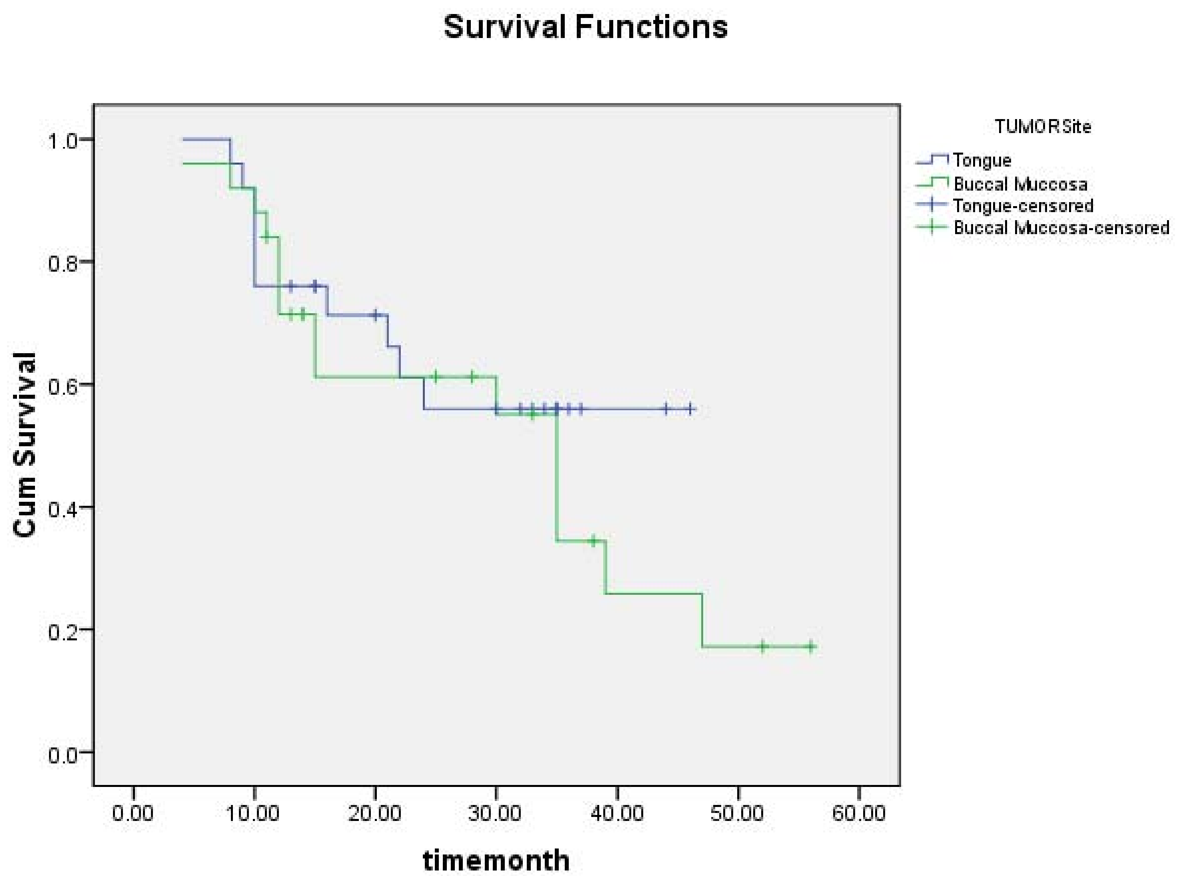

Figure 4.3 Survival rate of patients according to tumor site.

4.5.3. Kaplan-Meier survival analysis (KMSA) of cyclin D1 amplification in Tongue SCC:

According to Log Rank (Mantel-Cox) test the survival rate was not significantly different (*p*-value=0.147) for cyclin D1 amplification in tongue SCC patients. (Fig 4.3)

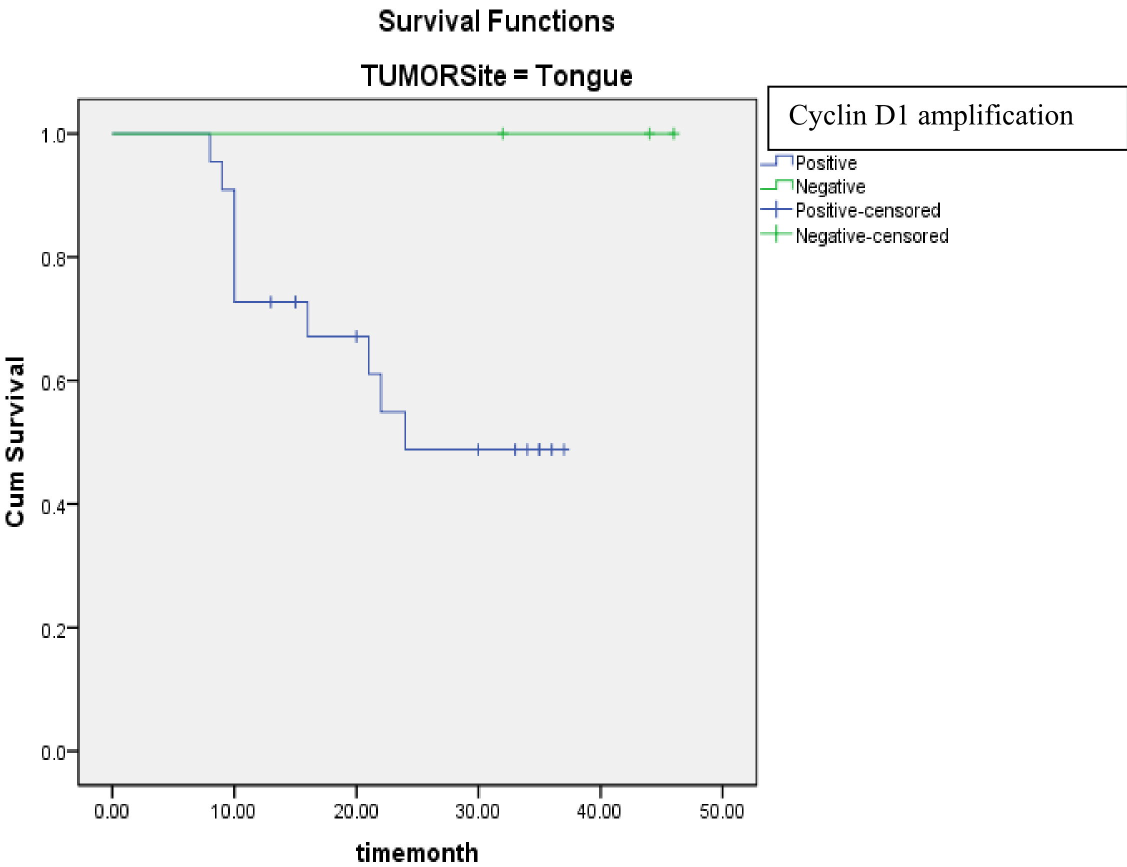

Figure 4.4 Survival rate of patients with tongue cancer (n=25) in relation to Cyclin D1 amplification.

4.5.4. Kaplan-Meier survival analysis (KMSA) of cyclin D1 amplification in Buccal mucosa SCC:

According to Log Rank (Mantel-Cox) test the survival rate was significantly different for cyclin D1 negatively amplified in buccal mucosa SCC patients . (*p*-value<0.001) (Fig 4.4)

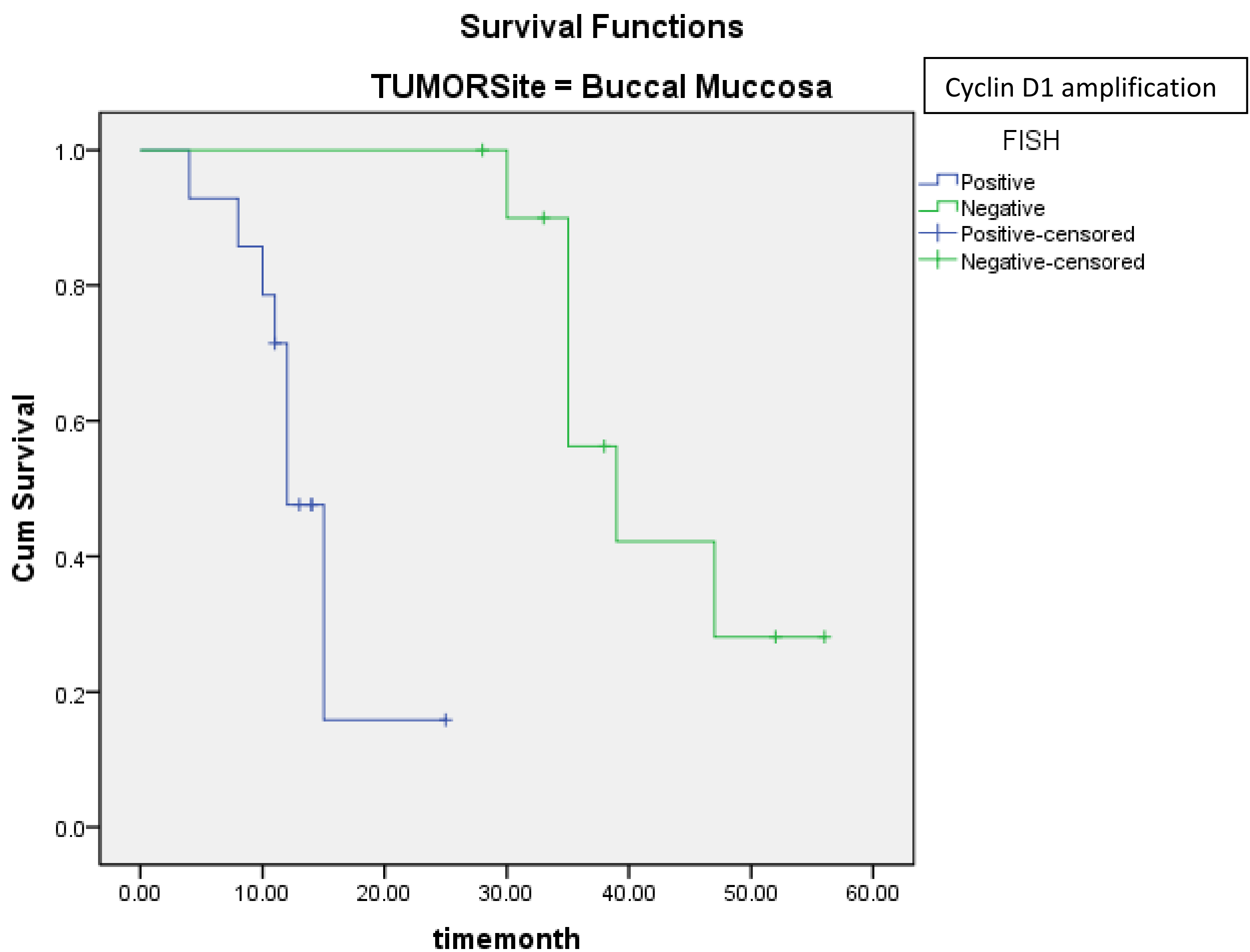

Figure 4.5 Survival rate patients with buccal mucosa cancer (n=25) in relation to Cyclin D1 amplification.

CHAPTER 5

DISCUSSION

In this study, we adopted FISH technique to detect cyclin D1 deregulation. The incidence of positive cyclin D1 amplification in overall cases was 36 cases (72%) which is similar to that in previous reports, Callender et al. (1994) and Kyomoto et al. (1997).

5.1. Sociodemographic characteristic:

The sample size of this study is relatively small as compared to other studies reported in the literature. Choi et al. (2006). RF de Araújo Júnior et al. (2008). In the current study, 50 cases were selected from year 2004 until 2010. Costello and Osborne (2005) tested the effect of sample size on the results of factor analysis reporting that larger samples tend to produce more accurate solutions.

5.1.1. Age:

The mean age in the current study was 60 years, in which 80% of the patients were in the 5th- 9th decade of life. This result concurs with previous studies that reported OSCC as a disease of older age groups in Malaysia (Ramanthan & Lakshimi 1976, Ng et al. 1985; Siar et al. 1990 and Ng & Siar 1997). While Burzynski et al. 1992 reported the incidence of oral cancer in patients less than 40 years of age about 3%. In the present study, there were three cases of young patients below 40 years old.

The present study showed that association of cyclin Dl amplification statistically insignificant with age for both tongue and buccal mucosa SCC. There is inequal distribution of age groups for tongue, therefore, the association between CD1 amplification and age cannot be confidently concluded separately for each group However, if considered as a whole group, age cannot be positively correlated with CD1 amplification. (i.e. we cannot say that the older the patient, the higher CD1 amplification).

5.1.2. Gender:

In the present study there are more female than male with a female to male ratio of 1.7:1. Previous local studies reported by Ng et al. (1985) and Siar et al. (1990) reported an overall female preponderance although the F:M ratio was smaller 1.1:1 and 1.5:1 respectively. Data from local studies have revealed that frequency of certain anatomical site involvement of OSCC vary with gender distribution (Ramanathan & Lakshimi 1976; Ng et al.1985). Generally, female preponderance was noticed in SCC of the buccal mucosa while male preponderance was found in SCC of the tongue. Thus, the distinct difference in the F: M ratio observed may be attributed to habits that predisposed to OSCC, as the present study showed. No significant statistical association between cyclin D1 amplification with gender.

5.1.3. Ethnicity:

Comparison among the racial differences of OSCC in previous studies showed that Indians were the predominant group (Ramanathan & Lakshimi 1976; Ng et al.1985, Siar et al. 1990). This distinct pattern of racial distribution of OSCC was also observed in the current study. Whereby, the Indian ethnic group represents 68% of total study sample.

Studies showed that the frequency of anatomical site involvement in OSCC also varies with the ethnic group (Ramanathan & Lakshimi 1976; Ng et al.1985). The dominant site was found to be the buccal mucosa among Indians while tongue was the most frequent site for Chinese samples (Ramanathan & Lakshimi 1976; Ng et al.1985, Siar et al. 1990). For the Malays, studies showed that tongue and buccal mucosa were equally dominant as reported by Ramanathan & Lakshimi (1976). In the current study, the dominant sites for both Indian and Malays were buccal mucosa 65%, 70% respectively while for Chinese the tongue showed 84%. However, in the present study, amplification of cyclin Dl has no significant statistical association with ethnicity.

5.2. Clinicopathological characteristic:

5.2.1. Tumor site:

Study sample consist of equal number of cases for both tongue and buccal mucosa. The positive amplification of cyclin D1 was recorded in 88% of tongue SCC cases and 54% of buccal mucosa SCC. A study by Fujii et al. (2001) coincided our result by recording positive amplification of cyclin D1 in 56.5% of tongue SCC.

Tongue and buccal mucosa SCC represent a different biological subentities, etiology as well as genetics and these differences should be considered in the management of OSCC (Kannan et al. 1997). There are differences in the effectiveness of the molecular pathway which participate in the development of tongue and buccal mucosa SCC (Sathyan et al. 2006).

The result of this study showed a significant difference between the amplification of cyclin D1 in tongue and buccal mucosa SCC (p-value=0.012), and this sugests that may be differences in the behavior of squamous cell carcinoma in tongue and buccal mucosa.

5.2.2. Tumor depth:

In the present study, the cut-off point of 5mm was used as various studies had considered tumor depth as a predictive factor for nodal metastasis and survival rate (Al-Rajhi et al. 2000, Gonzalez-Moles et al. 2002 and O-charoenrat et al. (2003). Okamoto et al. (2002) and Myo et al. (2005) found that tumor depth more than 4mm were significantly predictive for Cyclin D1 positively amplified.

This study revealed that 92% of tongue SCC cases and 96% of buccal mucosa SCC have tumor depth of 5 mm or more. This findings are in accordance with the findings of other studies that showed higher percentage of OSCC cases with tumor depth more than or equal to 5mm. (Gonzalez-Moles 2002, Kane et al. 2006).

For tongue SCC, there was a significant statistical association between the amplification of cyclin D1 and tumor depth of 5mm or more. Cyclin Dl positivity was identified in 22 (95.7%) cases of tongue SCC with tumor depth more than or equal to 5 mm, while none were identified with tumor depth less than 5 mm. This result is similar to that reported by Wang et al. (2006). There is no significant association between amplification of cyclin Dl and tumor depth in buccal mucosa cases.

5.2.3. Pattern of invasion:

Analyzing the pattern of invasion, the current study noted that the majority of cases (88%) were non-cohesive type. The same trend was observed in a western study by Spiro et al. (1999) who found that 61% of OSCC patients had non-cohesive pattern of invasion. Other studies have also reported results showing higher percentage of OSCC with non-cohesive type of pattern of invasion (Miyamoto et al. 2003; Kane et al. 2006; Boey 2002; Abdul Jalil 2003).

The current study showed no significant difference between pattern of invasion and cyclin D1 amplification for both tongue and buccal mucosa SCC. Shiraki et al. (2005) had also reported no significant association between overexpression of cyclin Dl and pattern of invasion in OSCC

A study conducted by Myo et al. (2005) showed that cyclin D1 numerical aberrations is always associated with overexpression of cyclin D1 protein. Thus, overexpression of this protein may affect the phenotypes of cancer cells. Cyclin D1 encodes an important regulatory protein that promotes cell cycle progression by activating CDK 4 and 6. However, recent studies have indicated that cyclin D1 affects the activity of various non-CDK-dependent cellular transcription factors such as estrogen, DMP1, STAT3 and BETA2/NeuroD (Lamb 2003).

It is not clear how overexpression or amplification of cyclin D1 protein affects the invasive and metastatic behavior of cancer cells, thus, its CDK-independent biological activities that may contribute to the acquisition of the invasive ability and metastatic potential of tumor cells.

However, overexpression of other oncogenes on the 11q13 amplicon, such as *EMS1* and *TAOS1*, may also contribute to the development of invasive and metastatic characteristics of cancer cells (Huang et al. 2002). Therefore, this issue is still a matter of controversy and further investigations are required.

5.2.4. pT:

In a study conducted by Chheitri et al. (2000), 59% of OSCC cases had tumor size of T1 and T2 and 41% had tumor size of T3 and T4. Similarly, Kuo et al. (1999) and Shraki et al. (2005) reported higher percentage of T1 and T2 compared to T3 and T4.

The current study had more OSCC with T1 and T2 tumor size (66%) as compared to T3 and T4 (34%). Nevertheless, tumor size has not been particularly effective in predicting the metastasis and survival rate as reported by Gonzales-Moles et al. (2001), Kane et al. (2006) and Woolgar (2006).

The present study showed significant statistical difference between cyclin Dl positivity amplification and tumor greatest dimension for tongue SCC cases, while for buccal mucosa SCC cases there is no significant difference statistically. This finding was completely opposite with a study done by Fujii (2001) in which cyclin Dl amplification was not significant with tumor size in tongue SCC.

5.2.5. pN:

The present study showed that (44%) of tongue SCC and (64%) of buccal mucosa SCC had positive lymph node. This findings is in contrast with what was reported by Woolgar (2006) that nodal metastasis were diagnosed histologically in 59-64% of SCC of the tongue compared to 22% of buccal mucosa SCC.

Nevertheless, the anatomical site is not the sole factor affecting the distribution of metastatic lymph nodes, as socioeconomic constraint such as delay in diagnosis and seeking treatment may affect the possibility of metastasis. A study in India done by Ghoshal et al. (2006) involving only the buccal mucosa revealed that 62% patients with OSCC had lymph node metastasis. Possible that more buccal mucosa cancer patients present late.

The current study showed that amplification of cyclin Dl was seen in 90.9% and 68.8% for tongue and buccal mucosa cases respectively with lymph node metastasis, and 85.8% and 33.3% for tongue and buccal mucosa cases respectively with non-metastatic lymph nodes. Thus, this association was statistically not significant which leads to the inability to reject the null hypothesis.

Fortin et al. (1997) did not observe any correlation between lymph node status and cyclin D1 amplification in patients with oral and oropharyngeal carcinomas. Fujii (2001) also found there was no correlation between cyclin Dl amplification and lymph node involvement in tongue SCC.

The studies conducted by Miyamoto R et al. (2002) and Myo K et al. (2005) on FISH technique utilized fresh fine needle aspirated biopsies and that might be the reason for the significant results obtained (Cyclin D1 numerical aberration were observed significantly associated with the presence of lymph node metastasis), whereas this study used paraffin-embedded tissue which was thought to be less feasible.

5.2.6. pTNM staging:

There are no reports found on pTNM stage of OSCC patients in Malaysia. Most studies in Malaysia involved clinical stage TNM as one of the parameters (Boey 2002, Abdul Jalil 2003, Lee 2006). This could be due to limitation of surgically resected cases at the diagnostic laboratory in previous years. The current study on surgically resected tumors revealed that the majority of the cases (64%) were pathological stages III and IV and the remaining cases (36%) were stages I and II. This finding is similar to a recent study done on Indian the population by Ghoshal et al. (2006), which showed that only 20% OSCC patients were in stages I and II and the remaining majority of 80% were in stages III and IV disease.

The current study showed that amplification of cyclin Dl has no significant statistical association with pTNM stage. The present finding is in contrast with a study done by de Vincente et al. (2002) which showed that overexpression of cyclin Dl was found to be positively correlated with advanced pathological tumor stage of SCC in oral cavity.

Michalides et al. (1995) and Bartkova et al. (1995) reported no association of cyclin D1 amplification with tumor stage in OSCC which supports the finding of the current study. However, Fujii et al. (2001) who studied the amplification of cyclin D1 only in tongue SCC reported that there is no correlation between the amplification of cyclin D1 and the associated tumor stage. More recent studies done

by Kuo et al. (2002) and Wang et al. (2006) reported that there is no correlation between positive expressions of cyclin D1 with clinical stage in OSCC.

5.2.7. Modified Broder's grading:

The present study revealed that the most frequently encountered type of OSCC according to modified Broder's grading was moderately differentiated SCC. Only 16% of the samples were poorly differentiated, all of which were from tongue SCC.

In different studies in Malaysia, the well-differentiated type was seen more commonly than the moderately differentiated type while the current study showed the highest prevalence being the moderately differentiated type. Siar et al. (1990) reported that well differentiated OSCC in 81.1% of the cases, 17.9% moderately differentiated and only 1% of poorly differentiated cases. Boey (2002) reported that most 51.9% and 33.3% of his study samples were well differentiated and moderately differentiated respectively and the remaining 14.8% of were poorly differentiated.

The current study coincides with most studies that reported a minority of OSCC patients with poorly differentiated type (Chhetri et al. 2000, Amaral et al. 2004, Rodolica et al. 2005). Kane et al. (2006) also reported higher percentage of moderately differentiated OSCC cases compared to poorly differentiated.

This study showed that there was no statistical significant association between cyclin Dl amplification and tumor grading. This is consistent with other studies done by

Kuo et al. 1999, Nakahara et al. (2000), de Vincente et al. (2002) and Wang et al. (2006) which stated that overexpression of cyclin Dl was not associated with histological grading in OSCC. However, Bova et al. (1999), Miyamoto et al. (2002) and Angadi & Lrishnapallai (2007) found that an amplification of cyclin D1 was associated with increased tumor grade in OSCC.

5.3. Survival rate:

In the present study, Kaplan-Meier survival analysis (KMSA) was performed and for cyclin D1 negative patient recorded a survival rate of 57.1%. This is considerably lower when compared to the study of Fujii et al. (2001) where the 5-year disease-free survival rate was 80.0% for negative patients, a result which was significantly better for cyclin D1 amplification negative patients. The reason for the difference in the result is that Fujii et al. (2001) studied only the tongue SCC whereas in the present study both tongue and buccal mucosa SCC were included and besides that, this study considered over-all survival rate instead of disease-free survival rate due to lack of information with regards cause of death. However, the Log Rank (Mantel-Cox) test for the current study resulted in a significant association between survival rate and cyclin D1 positive and cyclin D1 negative overall patients (p=0.009) and significant association between survival rate and cyclin D1 positive and cyclin D1 negative in buccal mucosa patients (p-<0.001).

The KMSA analysis based on tumor site was performed where the survival rate was 60% for tongue SCC and 40% for buccal mucosa SCC patients. The Log Rank (Mantel-Cox) test did not show any significant association between survival rate and cyclin D1 amplification for tongue SCC. This was in contrast to Sathyan et al. (2006) who studied the overexpression of cyclin D1 in tongue SCC. However, there was significant result observed between survival rate and cyclin D1 amplification in buccal mucosa SCC which was similar to Liu et al. (2004) who studied the overexpression of cyclin D1 in buccal SCC.

5.4. Limitation of the study:

The main setback of this study was the limited size of the sample. We were constrained by the availability of surgical specimens in our centre (Oral Pathology diagnostic archive at the Faculty of Dentistry, University of Malaya). Time constraint did not allow us to seek joint collaboration with other centres to increase the sample size.

CHAPTER 6

CONCLUSION, IMPLICATION and RECOMMENDATIONS

6.1. Conclusion:

In summary, the results of this study demonstrated that:

1. The amplification of cyclin D1 was identified in 72% (36) of total OSCC cases and the majority were tongue SCC (22).
2. Amplification of cyclin D1 in tongue SCC cases was significantly associated with only tumor depth (≥5 mm) and tumor greatest dimension.
3. No siginifcant association between amplification of cyclin D1 in buccal mucosa SCC with any of the sociodemographic and clinicopathological parameters.
4. Survival rate was only significantly better for cyclin D1 negative amplification of buccal mucosa SCC.

6.2. Implication of the study

These results indicate that cyclin D1 has a potential to be recognized as one of the promising prognostic markers in cancer. If cyclin D1 can be proven as equally reliable as other histopathological prognostic indicators, then a serum assay of cyclin D1 has a potential to be of great value for the surgeons to set a better and more accurate plans. Therefore, further studies with larger sample size are strongly recommended in order to confirm the significance of cyclin D1as a prognostic marker.

6.3. Recommendation

Based on the current study, it is recommended that:

- Larger sample size should be studied to validate the potential of cyclin D1 as the prognostic factor as it would give a more definitive result on the relationship of cyclin D1 amplification with the sociodemographic, clinicopathological parameters.
- Patients with good record data are included in the future studies as this study suffers from the lack of data on the cause of death and status of patients until last follow up.
- CDK independent pathway have to be investigated.
- Further wider studies to evaluate FISH technique as a routine investigation technique with estimating the cost value.

<u>References</u>

Abdul Jalil A. (2003). Comparison of expression of Ki-67 and PCNA at the tumor invasive front of oral squamous cell carcinoma. Unpublished master's thesis. University of Malaya, Kuala Lumpur.

Allison, P., Locker, D., & Fiene, JS. (1998). The role of diagnostic delays in the prognosis of oral cancer a review of literature. *Oral Oncol*, *34*(3), 161-170.

Altieri, A., Bosetti, C., Gallus, S., Franceschi, S., Dal Maso, L., Talamini, R., et al. (2004). Wine, beer and spirits and risk of oral and pharyngeal cancer: a case-control study from Italy and Switzerland. *Oral Oncol, 40,* 904-909.

Al-Rajhi, N., Khafaga, Y., El-Husseiny, J., Saleem, M., Mourad, W., Al-Otieschan, A., et al. (2000). Early stage carcinoma of oral tongue: prognostic factors for local control and survival, *Oral Oncol*, *36(6),* 508-14.

Amaral, T.M.P., Da Silva Freire, A.R., Carvalho, A.L., Pinto, C.A. & Kowalski, L.P. (2004). Predictive factors of occult metastasis and prognosis of clinical stages I and II squamous cell carcinoma of the tongue and floor of the mouth. *Oral Oncol, 40*, 780-786.

Angadi, P.V., & Krishnapallai, R. (2007). Cyclin D1 expression in oral squamous cell carcinoma and verrucous carcinoma: correlation with histological differentiation. *Oral Surg Med Oral Pathol Oral Radiol Endod, 103,* 30-35.

Ayo-Yusuf, O.A., Swart, T.J.P., & Pickworth, W. (2004). Nicotine delivery capabilities of smokeless tobacco products and implications for control of tobacco dependence in South Africa. *Tob Control, 13,* 186-189.

Balaram, P., Sridhar, H., Rajkumar, T., Vaccarella, S., Herrero, R., Nandakumar, A., et al. (2002). Oral cancer in southern India: the influence of smoking, drinking, paan-shewing and oral hygiene. *Int J Cancer, 98,* 440-445.

Baldin, V., Lukas, J., Marcote, M.J., Pagano, M., & Draetta, G. (1993): Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev*, *7,* 812-821.

Barrera, J.E., Ai, H., Pan, Z., Meyers, A.D. & Varella-Garcia, M., (1998). Malignancy detection by molecular cytogenetics in clinically normal mucosa adjacent to head and neck tumors. *Arch Otolaryngol Head Neck Surg, 124*, 847-851.

Bartek, J., Lukas, J. & Bartkova, J. (1999). Perspective: Defects in cell cycle control and cancer. *J Pathol, 187,* 95-99.

Bartkova, J., Lukas, J., Muller, H., Strauss, M., Gusterson, B., & Bartek, J. (1995). Abnormal of cyclin D1 expression and G1 regulatory in human head and neck cancer. *Cancer Res, 55,* 949-956.

Batsakis, JG. (Eds). (2003). *Clinical Pathology of Oral Cancer. In JP Shah, NW Johnson, JG Batsakis. OralCancer,* London; Martin Dunitz, an imprint of The Taylor & Francis Group.

Binnie, V.H., & Rankin, K.V. (1983). Etiology of oral squamous cell carcinoma. *J Oral Pathol, 12,* 11-29.

Blot, J.W., Mclaughlin, J.K., Winn, D.M., Austin, D.F., Greenberg, R.S., Preston-Martin, S., et al. (1988). Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res., 48,* 3282-3287.

Boey, W. (2002). *Expression of p53 and PCNA at the tumor invasive front of oral squamous cell carcinomas in buccal mucosa.* Kuala Lumpur, Malaysia : Faculty of dentistry, University Malaya.

Boffeta, P., Mashberg, A., Winkelmann, R., & Garfinkel, L. (1992). Carcinogenic effect of tobacco smoking and alcohol drinking on anatomic sites of the oral cavity and orpharynx. *Int J Cancer, 52,* 530-533.

Bova, R.J., Quinn, D.I., Nankervis, J.S., Cole, I.E., Sheridan, B.F., Jensen, M.J., et al. (1999). Cyclin D1 and p16 expression predict reduced survival in carcinoma of the anterior tongue. *Clin Cancer Res,* 5, 2810-2819.

Broders, A.C. (1920). Squamous cell epithioma of the lip: A study of five hundred and thirty seven cases. *J Am Med Assoc., 74*(10), 656-664.

Bryne, M. (1998). Is the invasive front of an oral carcinoma the most important area for prognostication? *Oral Dis, 4*(2)*,* 70-77.

Bryne, M., Jenssen, N., & Boysen, M.. (1995). Histologic grading in the deep invasive front of T1 and T2 glottic squamous cell carcinomas has high prognostic value. *Virchows Arch, 427,* 277-281.

Burim, R.V., Canalle, R., Takahashi, C.S., Tavares, D.C., Martinelli, A.d., Ade, L., et al. (2004). Clastogenic effect of ethanol in chronic and abstinent alcoholics. *Mutat Res, 560*(2)*,* 187-198.

Callender, T., El-Nagger, A.K., Lee, M.S., Frankenthaler, R., Luna, M.A., & Batsakis, J.G. (1994) PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer, 74,* 152–158.

Chhetri, D.K., Rawnsely, J.D., & Calcaterra, T.C. (2000). Carcinoma of the buccal mucosa. *Otolaryngology-Head Neck Surg, 123,* 566-571.

Cawson, R.A., & Odell, E.W., (2002). *Oral Cancer. Essentials of oral pathology and oral medicine*. Churchill Livingstone.

Chen, J.W., & Katz, R.V. (1990). Intraoral squamous carcinoma: epidemiological patterns in Connecticut from 1935-1985. *Cancer, 66,* 1288-1296.

Chhetri, D.K., Rawnsely, J.D., & Calcaterra, T.C. (2000). Carcinoma of buccal mucosa. *Otolaryngology Head Neck Surg, 123,* 566-571.

Chidzonga, M.M. (2005). Lip cancer in Zimbabwe. Report of 14 cases. *Int J Oral Maxillofac Surg, 34,* 149-151.

Choi, K.K., Kim, M.J., Yun, P.Y., Lee, J.H., Moon, H.S., Lee, T.R., et al. (2006). Independent prognostic factors of 861 cases of oral squamous cell carcinoma in Korean adults. *Oral Oncology, 42*(2)*,* 208–217.

Cordon-Cardo, C. (1995). Mutation of cell cycle regulators-biological and clinical implications for human neoplasia. *Am J of Pathol, 147,* 545-560.

Costello, A. B., & Osborne, J. W., (2005). Best practices in exploratory factor analysis: four recommendations for getting the most from your analysis, Practical Assessment, *Research & Evaluation, 10*(7).

Cremar, T., Tessin, D., Hopman, A.H., & Manuelidis, L. (1988). Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumour cells by in situ hybridization with chemically modified DNA probes. *Exp Cell Res, 176,* 199-220.

DeMarini, D.M. (2004). Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. *Mut Res, 567,* 447-474.

de Vicente, C., Herrero-Zapatero, J., Fresno, A., & López-Arranz, J.S. (2002). Expression of cyclin D1 and Ki-67 in squamous cell carcinoma of the oral cavity: clinicopathological and prognostic significance. *Oral Oncol, 38*(3)*,* 301-308.

de Visscher, J.G.A.M., & van der Waal, I. (1998). Etiology of cancer of the lip.*Int. J. Oral Maxillofac. Surg., 27*(3)*,* 199-203.

Dickenson, A.J., Currie, W.J.R., & Avery, B.S. (1995). Screening for syphilis in patients with carcinoma of the tongue. *Br J Oral Maxillofacial Surg, 33*(5)*,* 319-320.

Dodds, A.P., Rankin, K.V., Jones, D.L., & Seals, R,R, Jr. (1994). What are you doing about oral cancer? Report of a survey of texas dentist. *Texas Dent J, 114,* 39-41.

Dongpo, Cai., Vaughan, M., Latham Zhang, X., & Shapiro, G.I. (2006), Combined Depletion of Cell Cycle and Transcriptional Cyclin-Dependent Kinase Activities Induces Apoptosis in Cancer Cells. *Cancer Research, 66,* 9270-9280.

Dubrow, R. & Wegman, D.H. (1984). Cancer and occupation in Massachusetts: a death certificate study. *Am J Industrial Med, 6,* 207-230.

El-Naggar, A.K., Hurr, K., Hurr, V. Huff, G. L., Clayman, M. A., & Batsakis, J. G. (1996). Microsatellite instability in pre-invasive and invasive head neck squamous carcinoma. *Am J Pathol, 148*(6)*,* 2067-2072.

Emmerich, P., Jauch, A., Hofmann, M.C., Cremer, T. & Walt, H. (1989). Interphase cytogenetics in paraffin embedded sections from testicular germ cell tumor xenografts and in corresponding cultured cells. *Labs Invest, 61,* 235-242.

Farber, E. (1980). The sequential analysis of cancer development. *Adv Cancer Res, 1*(31), 125.

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumourigenesis. *Cell, 61,* 759-767.

Ferlito, A., Rinaldo, A., Robbins, K.T., Leemans, C.R., Shah, J.P., Shaha, A.R., et al. (2003). Changing concepts in the surgical management of the cervical node metastasis. *Oral Oncol, 39(5),* 429-435.

Fortin, A., Guerry, M., Guerry, R., Talbot, M., Parise, O., Schwaab, G., et al. (1997). Chromosome 11q13 gene amplifications in oral and oropharyngeal carcinomas: no correlation with subclinical lymph node invasion and disease recurrence. *Clin Can Res., 3*(9), 1609–1614.

Franceschi, S., Favero, A., Conti, E., Talamini, R., Volpe, R., Negri, E., et al. (1999). Food groups, oils and butter, and cancer of the oral cavity and pharynx. *Br J Cancer, 80,* 614-620.

Francesco, R., Schirosi, L., Bettelli, S., Bigiani, N., & Curatola, C. (2008). FISH analysis in cell touch preparations and cytological specimens from formalin-fixed fetal autopsies. *Diagnostic Cytopathology, 36*(9), 633–636.

Fukano, H., Matsuura, H., Hasegawa, Y., & Nakamura, S. (1997). Depth of invasion as a predictive factor for cervical lymph node metastasis in tongue carcinoma. *Head & Neck, 19,* 205-210.

Fujii, M., Ishiguro, R., Yamashita, T., & Tashiro, M. (2001). Cyclin D1 amplification correlates with early recurrence of squamous cell carcinoma of the tongue. *Cancer Letters, 172,* 187–192.

Ghoshal, S., Mallick, I., Panda, N., & Sharma, S.C. (2006). Carcinoma of buccal mucosa: Analysis of clinical presentation, outcome and prognostic factors. *Oral Oncol, 42,* 533-539.

Giwereman, A., Hopman, A.H.N., Ramaekers, F.C. & Skakkebaek, N. E. (1990). Carcinoma in situ of the testis. Detection of malignant germ cells in seminal fluid by means of in situ hybridization. *Am J Pathol, 136,* 497-502.

Goldenberg (2002). A risk factor for oral and oropharyngeal cancer. *Oral Oncol, 38,* 646-649.

Gonzalez-Moles, M.A., Esteban, F., Rodriguez-Archilla, A., Ruiz-Avila, I. & Gonzalez-Moles, S. (2002). Importance of tumour thickness measurement in prognosis of tongue cancer. *Oral Oncol, 38*(4), 394-397.

Gonzalez-Moles, M.A., Galindo, P., Gutierrez-Fernadez, J., Sanchez-Fernandez, E., Rodriguez-Archikka, A., Ruiz-Avilla, et al. (2001). P53 protein expression in oral squamous cell carcinoma, survival analysis. *Anticancer Res., 21,* 2889-2894.

Greene, F. & Page, D. (2002). *Cancer Staging Manual Sixth Edition*. New York, Springer-Verlag.

Grunfeld, E. (2005). Health Services and Outcomes Research. *Cancer Care Nova Scotia, 2,* 3.

Guo, Y., Yang, K., Harwalkar, J., Nye, J.M., Mason, D.R., Garrett, M.D., et al. (2005): Phosphorylation of cyclin D1 at Thr 286 during S phase leads to its proteasomal degradation and allows efficient DNA synthesis. *Oncogene, 24,* 2599-2612.

Gupta, P.C., & Ray, C.S. (2004). Epidemiology of Betel Quid Usage. *Ann Acad Singapore, 33,* 31S-36S.

Gupta, P.C., & Nandakumar, A. (1999). Oral cancer scene in India. *Oral Dis, 5*(1)*,* 1-2.

Hackel, C., & Varella-Garcia, M. (1997).Interphase cytogenetics using fluorescent in situ hybridization: an overview of its application to diffuse and solid tissue. *Braz J Genet; 10,* 97-106.

Gabriel, H.E., Liu, Z., Crott, J.W., Choi, S.W., Song, B.C., Mason, J.B., & Johnson, E.J. (2006). A comparison of carotenoids, retinoids and tocopherols in the serum and buccal mucosa of chronic cigarette smokers versus nonsmokers. *Cancer Epi Bio Prev, 15,* 993-999.

Helliwell, T.R., & Woolgar, J.A. (2000). *Minimum data set for head and neck carcinoma.* London: Roal College of Pathologist.

Hemmer, J., & Prinz, W. (1997). Comparison of DNA flow cytometry and fluorescence in situ hybridization with a set of 10 chromosome-specific DNA probes in four head and neck carcinomas. *Cancer Gene Cytogenet, 97,* 35-38.

Hindle, I., Downer, M.C., Moles, D.R., & Speight, P.M. (2000). Is alcohol responsible for more intra-oral cancer? *Oral Oncol, 36,* 328-333.

Hollows, P., McAndrew, P.G., & Perini, M.G. (2000). Delays in the referral and treatment of oral squamous cell carcinoma. *Br Dent J*, *188*(5)*,* 262-265.

Hopman, A.H.N., Poddighe, P.J., Smeets, A.W., Moesker, O., Beck, J.L., Vooijs, G.P., et al. (1989). Detection of numerical chromosome aberrations in bladder cancer by in situ hybrization. *Am J Pathol, 135,* 1105-1117.

Hopman, A.H.N., Ramaerkers, F.C.S., Raap, A.K., Beck, J.L., Devilee, P., van der Ploeg, M., et al. (1988). In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumours. *Histochem, 89,* 307-316.

Horowitz, A.M., Drury, T.F., Goodman, H.S., & Yellowitz, J.A. (2000). Oral pharyngeal cancer prevention and early detection. Dentist's opinions and practices. *Oral Am Dent Assoc; 131,* 453-462.

Huang, X., Gollin, S.M., Raja, S., & Godfrey, T.E. (2002). High-resolution mapping of the 11q13 amplicon and identification of a gene, TAOS1, that is amplified and overexpressed in oral cancer cells. *Proc Natl Acad Sci U S A., 99*(17), 11369-74.

Hyunmin, Yi., Li-Qun, Wu., Ghodssi, R., Rubloff, G.W., Payne, G.F., & Bentley, W.E. (2005). Signal-Directed Sequential Assembly of Biomolecules on Patterned Surfaces. *Langmuir, 21*(6), 2104–2107.

Israel, H.A. (1986). Early squamous cell carcinoma of the mouth part I: Clinical evaluation. *Compend Contin Educ Dent, 7,* 563-546, 566-570.

John, W.W. (2007). *Oral cancer diagnosis.* management and rehabilitation.

Johnson, N.W. (2001). Tobacco use and oral cancer: A global perspective. *J Dent Edu, 65,* 328-339.

Johnson, N.W. (Eds) 1999. *Oral cancer. London: FDI World Dental Press Ltd.*

Johnson, N.W., & Warnakulasuriya, K.A.A.S. (1993). Epidemiology and aetiology of oral cancer in the United Kingdom. *Comm Dent Health,.10,* 13-29.

Kane, S.V., Gupta, M., Kakade, A.C., & D'Gruze, A. (2006). Depth of invasion is the significant histological predictor of subclinical cervical lymph node metastasis in early squamous cell carcinomas of the oral cavity. *J. Cancer Surg*., *32,* 1-9.

Kannan, S., Tahara, H., Yokozaki, H., Mathew, B., Nalinakumari, KR., Nair, M.K. et al. (1997). Telomerase activity in premalignant and malignant lesions of human oral mucosa. *Cancer Epidemiol Biomarkers Prev., 6*(6), 413-20.

Kerr, J.F.R. (1971). Shrinkage necrosis: a distinct mode of cellular death. *J Path, 105,* 13-20.

Kerr, J.F.R., Wyllie, A.H., & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer, 26,* 239-257.

Ko, Y.C., Huang, Y.L., Lee, C.H., Chen, M.J., Lin, L.M., & Tsai, C.C. (1995). Betel quid chewing, cigarette smoking and alcohol sonsumption related to oral cancer in Taiwan. *J oral Pathol Med, 24,* 450-453.

Kobayashi, I., Shima, K., Saito, I., Kiyoshima, T., Matsuo, K., Ozeki, Set al. (1999). Prevalence of Epstein-Barr virus in oral squamous cell carcinoma. *J Pathol, 189,* 34-39.

Kristensen, G.B., Abeler, V.M., Bjørn, Risberg, Claes Tropé, & Magne Bryne. (1999). Tumor size, depth of invasion and grading of the invasive front are the main prognostic factors in early squamous cell cervical carcinoma. *Gynecologic Oncolog,74,* 245-251.

Krogh, P. (1990). The role of yeasts in oral cancer by means of endogenous nitrosation. *Acta Odontol Scand., 48*(1), 85-88.

Kuo, M.Y.P., Lin, C.Y., Cheng, S.J., & Chiang, C.P. (1999). Expression of cyclin D1 is correlated with poor prognosis in patients with areca quid chewing related oral squamous cell carcinoma in Taiwan. *J. Oral Pathol Med., 28,* 165-169.

Kuo, M.Y.P., Hsu, H.Y., Kok, S.H., Kuo, R.C., Yang, H., Hahn, L., et al. (2002). Prognostic role of p27 expression in oral squamous cell carcinoma in Taiwan. *Oral Oncol*, *38,* 172-178.

Kyomoto, R., Kumazawa, H., Toda, Y., Sakaida, N., Okamura, A., Iwanaga, M., et al. (1997). Cyclin-D1-gene amplificaiton is a more potent prognostic factor than its protein over-expression in human head-and-neck squamous cell carcinoma. *Int J Cancer.,74,* 576–581.

Lamb, J., Ramaswamy, S., Ford, L., Contreras, B., Martinez, R.,Frances, S., et al. (2003). A Mechanism of Cyclin D1 Action Encoded in the Patterns of Gene Expression in Human Cancer .*Cell*, *114*(3), 323-334.

Lee, J.J., Hung, H.C., Cheng, S.J., Chen, Y.J., Chiang, C.P., Liu, B.Y., et al. (2006). Carcinoma and dysplasia in Taiwan: prevalence and risk factors. *Oral Pathol Oral Med Periodontal, 101,* 472-480.

Lee, C.N. (2006). *Immunohistochemical study of proliferative and apoptotic activity at the tumor invasive front in oral squamous cell carcinoma*. Kuala Lumpur, Malaysia: Faculty of Dentistry, University of Malaya. (Dissertation).

Lengauer, C., Speicher, M.R., Susanne, P., Jauch, A., Masafumi, T., Ramaiah, N., et al. (1993). Chromosomal bar codes produced by multicolor fluorescence in situ hybridization with multiple YAC clones and whole chromosome painting probes. *Hum Mol Genel, 2,* 505.

Levi, F., Pasche, C., La Vecchia, C., Lucchini, F., Franceschi, S., & Monnier, P. (1998). Food groups and risk of oral and pharyngeal cancer. *I Int J Cancer, 77,* 705-709.

Levy, D.T., Mumford, E.A., Michael K.C., Elizabeth, A., Gilpin, G.A., Giovino, A., et al. (2004). The relative risks of low-nitrosamine smokeless tobacco product compared with smoking cigarettes: estimates of panel of experts. *Cancer Epidemiol Biomarkers Prev, 13*(12), 2035-2042.

Liu, C.J., Chang, K.W., Chao, S.Y., Kwan, P.C., Chang, S.M., Yen, R.Y., et al. (2004). The molecular markers for prognostic evaluation of areca-associated buccal squamous cell carcinoma. *J Oral Pathol Med, 33*, 327–334.

Llewellyn, C.D., Johnson, N.W., & Warnakulasuriya, K.A. (2001). Risk factors for squamous cell carcinoma of the oral cavity in young people. A comprehensive literature review. *Oncol Oncol, 37,* 401-418.

Machlin, L.J., & Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J, 1,* 441-445.

Macluskey, B.O., Piffkò, J., & Bànkfalvi, A. (2004). Prognostic and predictive factors in oral squamous cell cancer: important tools for planning individual therapy. *Oral Oncology, 40*(2), 110–119.

Mahazir, S., Malik, R., Maria, M., Kanwal, A., Farida, M., Atif, M., et al. (2006). Socio-demographic correlates of betel, areca and smokeless tobacco use as a high

risk behavior for head and neck cancers in a squatter settlemenet of Karanchi, Pakistan. *Subs Abus Treat Prev Policy, 1,* 1-6.

Mashberg, A. (2000). Diagnosis of early oral and oropharyngeal squamous carcinoma: obstacles and their amelioration. *Oral Oncol, 36,* 253-255.

Massano, J., Regateiro, F.S., Januário, G., & Ferreira, A. (2006). Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 102,* 67-76.

McCullough, M., Jaber, M., Barrett, A.W., Bain, L., Speight, P.M., & Porter, S.R.. (2002). Oral yeast carriage correlates with presence of oral epithelial dysplasia. *Oral Oncol., 38*, 391-393.

Meyer, C.A., Jacobs, H.W., & Lehner, C.F. (2002). Cyclin D–cdk4 is not a master regulator of cell multiplication in *Drosophila* embryos. *Curr. Biol., 12,* 661-666.

Meyer, I., & Abbey, L.M. (1970). The relationship of syphilis to primary carcinoma of the tongue. *Oral Surg Oral Med Oral Pathol., 30,* 678-681.

Michalek, A.M., Mahoney, M.C., McLaughlin, C.C., Murphy, D., & Metzger, B.B. (1994). Historical and contemporary correlates of syphilis and cancer. *Int J Epidemiology, 23,* 381-385.

Michalides, R., Van, V.N., Hart, A., Loftus, B., Wientjens, E., & Blam, A. (1995). Overexpression of cyclin D1 correlates with recurrence in a group of forty seven operable squamous cell carcinoma of the neck and head. *Cancer Res., 55,* 975-978.

Miller, C.S., & Johnstone, B.M. (2001). Human papillomavirus as a risk factor for oral squamous cell carcinoma: A meta-analysis, 1982-1997. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod., 91*, 622-635.

Miyamoto, R., Uzuwa, N., Nagaoka, S., Hirata, Y., & Amagasa, T. (2003). Prognostic significance of cyclin D1 amplificarion and overexpression in oral squamous cell carcinoma. *Oral Oncol., 39,* 610-618.

Miyamoto, R., Uzuwa, N., Nagaoka, S., Nakakuki, K., Hirata, Y., & Amagasa, T. (2002). Potential marker of oral squamous cell carcinoma aggressiveness detected by Fluorescence in Situ Hybridization in fine-needle aspiration biopsies. *Cancer., 95*(10), 2152-9.

Moller, H. (1989). Changing incidence in of cancer of the tongue, oral cavity and pharynx in Denmark. *J Oral Path Med., 18,* 224-229.

Moore, S.R., Johnson, N.W., Pierce, A.M., & Wilson, D.F. (2000). The epidemiology of tongue cancer; a review of global incidence. *Oral Dis., 6,* 75-84.

Moulin, J.J., Mur, J.M., Pascal, W. J., Perreaux, P., & Quang, T.P. (1986). Oral cavity and laryngeal cancers among man-made mineral fiber production workers. *Scand J Work Enviro Hlth, 12,* 27-31.

Murphy, J.J. (2002). Cytogenetic and histologic findings in Wilms' tumor, *J Pediatr Surg 37,* 823–827.

Murray, A. (2004). Recycling the Cell Cycle Cyclins Revisited. *Cell, 116*(2), 221–234.

Myo, K., Uzawa, N., Miyamoto, R., Sonoda, I., Yuki, Y., & Amagasa, T. (2005). Cyclin D1 gene numerical aberration is a predictive marker for occult cervical lymph node metastasis in TNM Stage I and II squamous cell carcinoma of the oral cavity. *Cancer., 104*(12), 2709-16.

Nagy, K.N., & Sonkodi, I. (1998). The microflora associated with human oral carcinomas. *Oral Oncology., 34,* 304-308.

Nakahara, Y., Shintani, S., Mihara, M., Kiyota, A., Ueyama, Y., & Mastsumura, T. (2000). Alternation of Rb, p16 and cyclin D1 in the tumorigenesis of oral squamous cell carcinoma. *Cancer Lett., 160,* 3-8.

Nederlof, P.M., & Robinson, D. (1989). Three-color fluorescence in situ hybridization for the simultaneous detection of multiple nuclei acid sequences. *Cytomert, 10,* 20-27.

Negri, E., & Franceschi, S. (2000). Selected micronutrients and oral and pharyngeal cancer. *Int J Cancer, 86,* 122-127.

Neville, B.M., & Day, T.A. (2002). Oral cancer and precancerous lesions. *CA Cancer J Clin., 52,* 195-215.

Ng, K.H., Siar, C.H., Ramanathan, K., Murugasu, P., & Chelvarnayagam, P.I. (1985). Squamous cell carcinoma of the oral mucosa in Malaysia-any change? *Southeast Asian L Trop. Med. Pub. Hlth., 16*(4), 602-606.

Ng, K.H., & Siar, C.H. (1997).Oral cancer in Malaysia and national oral cancer control programme. *Dent J Malaysia, 18,* 48-51.

Nowell, P.C. (1976). *The clonal evolution of tumor cell populations*. University of Pennsylvania, Philadelphia.

O-charoenrat, P., Pillai, G., Patel, S., Fisher, C., Archer, D., Eccles, S. et al. (2003). Tumor thickness predicts cervical nodal metastases and survival in early tongue cancer. *Oral Oncol, 39*(4), 386–90.

Ogden, G.R., & Macluskey, M. (2000). An overview of the prevention of oral cancer and diagnostic markers of malignant change: 1. Prevention. *Dent Update, 27,* 95-99.

Okamoto, M., Nishimine, M., Kishi, M., Kirita, T., Sugimura, M., Nakamura, M., et al. (2002). Prediction of delayed neck metastasis in patients with stage I/II squamous cell carcinoma of the tongue. *J Oral Pathol Med., 31*(4), 227-33.

Oliver, A.J., & John, F. (1996). Primary oral squamous cell carcinoma: A review of 92 cases. *Journal of Oral and Maxillofacial surgery, 54*(8), 949-954.

Ortiz, G.M., Pierce, Am., & Wilson, DF. (1996). Palatal changes associated with reverse smoking in Filipino women. *Oral Dis., 2*(3), 232-237.

Parkin, D.M., Pisani, P., & Ferlay, J. (1993). Estimate of the worldwide incidence of eighteen major cancers in 1985. *Int J Cancer., 54*(4), 594-606.

Patrikidou, A., & Harris, M. (2001). Comment on: S.S. Prime et al. "A review of inherited cancer syndromes and their relevance to oral squamous cell carcinoma" *Oral Oncology, 37*(1), 1–16.

Pickwell, S.M., & Schimelpfening, (1994). "Betel mania" Betel quid chewing by Cambodian women in the United States and its potential health effects. *West J Med, 160,* 326-330.

Pindborg, J.J., & Reichart, P.A. (1997). *Histological typing of cancer and precancer of the oral mucosa.* 2nd ed. Berlin, New York, Tokyo. Springer Verlag.

Platz, H., Fries, R., & Hudec, M. (1985). Retrospective DOSAK study on carcinomas of the oral cavity: results and consequences. *J Maxiilofac Surg, 13*(4), 147-152.

Platz, H., Fries, R., Hudec, M., Min Tjoa, A., & Wagner, R.R. (1983). The prognostic revalance of varios factors at the time of first admission of the patient. *J Maxillofac Surg, 11*(1), 3-12.

Popovtzer, A., & Shpitzer, K.T. (2004). Squamous cell carcinoma in oral tongue in younger patients. *Laryngoscope, 114,* 915-917.

Ramanathan, K., & Lakshimi, S. (1976). Oral carcinoma in Peninsular Malaysia: Racial variation in Indians, Malays, Chinese and Caucasians. *Gann Monograph on Cancer Research., 18,* 27-36.

Rankin, K.V., & Burznaski, N.J. (1999). Cancer curricle in U.S. dental schools. *J Cancer Educ, 14,* 8-12.

Reichart, P.A. (1999). Clinical management of selected oral fungal and viral infections during HIV-disease. *Int Dental J., 49,* 251-259.

RF de Araújo Júnior, CA., Galvão Barboza Naianne, K.C., Sergio Adrianne, B.M., & Antônio de Lisboa, L.C. (2008). Prognostic significance of the anatomical location and TNM clinical classification in oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal, 13*(6), 344-347.

Rodolico, V., Aragona, F., Cabibi, D., Di Bernardo, C., Di Lorenzo, R., Gebbia, N., et al. (2005).Overexpression of Cyclin D1 and interaction between p27 and tumor thickness predict lymph node metastasis occurrence in lower lip squamous cell carcinoma. *Oral Oncol., 40,* 207-213.

Rodu, & Jansson, C. (2004). Smokeless tobacco and oral cancer: a review of the risks and determinants. *Crit Rev Oral Biol Med, 15*(5), 252-263.

Rumboldt, Z., & Day, T. (2006), Imaging of oral cavity cancer: *Oral Oncology, 42* (9), 854–865.

Ryan, B. (2010). Applications of fluorescence in situ hybridization (FISH) in detecting genetic aberrations of medical significance. *Bioscience Horizons, 3*(1), 85-95.

Sancar, A. (1996). DNA excision repair. *Annu. Rev. Biochem, 65,* 43-81.

Sand, L.P., Jalouli, J., Larsson, P.A., & Hirsch, (2002). Prevalence of Epstein-Barr virus in oral squamous cell carcinoma, oral lichen planus and normal oral mucosa. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod., 93,* 586-592.

Sathyan, K.M., Sailasree, R., Jayasurya, R., Lakshminarayanan, K., Thomas, A., Nalinakumari, K.R., et al. (2006). Carcinoma of tongue and the buccal mucosa represent diVerent biological subentities of the oral carcinoma. *J Cancer Res Clin Oncol, 132,* 601–609.

Schildt, E.B., Eriksson, M., Hardell, L., & Magnuson, A. (1998). Oral snuff, smoking habits and alcohol consumption in relation to oral cancer in a Swedish xase-control study. *Int J Cancer, 77,* 341-346.

Schneeberger, C., Sabine, E., Herwig, S., Robert, U., & Robert, Z. (1998). A differential PCR system for the determination of CCND1 (Cyclin D1) gene amplification in head and neck squamous cell carcinomas. 34(4), 257-260.

Scully, C., & Bedi, R. (2000). Ethnicity and oral cancer. *Lancet Oncol., 1*(1), 37-42.

Scully, C., Field, J.K., & Tanzawa, H. (2000a). Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control, *Oral Oncol, 36,* 256–263.

Siar, C.H., Ng, K.H., Mah, C.F., & Ling, C.C. (1990). Oral squamous cell carcinoma in Peninsular Malaysia. *Asian Med. J., 33*(12), 697-703.

Silverman, S. (2001). Demographics and occurrence of oral and pharyngeal cancers. The outcome, the trends, the challenge. *JADA., 132,* 7-11.

Silververg, E., & Boring, C.C. (1995). Cancer Statistic 1995. *Cancer J ClinI, 40,* 9:26.

Silvio, K.H., Dante, A.M., & Norberto, N.S. (2006). Oral squamous cell carcinoma in a young patient. *Case report and literature review., 81*(3), 251-254.

Siriwardena, B.S., Tilakaratne, A., Amaratunga, E.A., & Tilakaratne, W.M. (2006). Demographic, etiological and survival differences of squamous cell carcinoma in the young and the old in Sri Lanka. *Oral Oncol., 42*(8), 831-836.

Smith, E.M., Hoffman, H.T., & Summersgill, K.S. (1998). Human papillomavirus and risk of oral cancer. *Laryngoscope., 108,* 1098-1103.

Snehal, G., & Jatin, P. (2005). TNM Staging of Cancers of the Head and Neck: Striving for Uniformity Among Diversity. *CA Cancer J Clin, 55,* 242-258.

Speight, P. M., & morgan, P.R. (1993). The natural history and pathology of oral cancer and precancer. *Community dental health, 10,* 1:71-8.

Spiro, R.H., Guillamondegui, O., Paulino, A.F., & Huvos, A.G. (1999). Pattern of invasion and margin assessment in patients with oral tongue cancer. *Head Neck.* 408-413.

Stahl, W., & Sies, H. (2005). Bioactivity and protective effects of natural carotenoids. *Biochemical et Biophy Acta, 1740*, 101-107.

Stanbridge, E.J., & Nowell, P.C. (1990). Origins of human cancer revisited. *CelI, 63,* 867-874.

Stephen, M., David, R., Dawn E., Sherianne, R., Peggy, L., & Chu, C. (2001). Oral Squamous Cell Cancer Risk in relation to alcohol consumption and alcohol dehydrogenase-3 genotypes. *Cancer Epidemiology Biomarkers & Prevention, 10,* 1137-1144.

Steward, B.W., & Kleihues, P. (2003). *Head and neck Cancer in World Cancer Report.* Bernard W Steward and Paul Kleihuse(eds). Lyon France. *IRAC Press.* 232-235.

Streilein, J.W. (1991). Immunogenetic factors in skin cancer. *New Engl J Med, 325,* 844-847.

Thomas, D.W., Seddon, S.V., & Shepherd, J.P. (1993). Systemic immunosuppression and oral malignancy: a report of a case and review of the literature. *Br J Oral Maxillofac Surg, 31*(6), 391-393.

Todd, R., Donoff, R.B., & Wong, D.T. (1997). The molecular biology of oral carcinogenesis: towards a tumor progression model *J Oral Maxillofac Surg*., 55, 613-623.

Toshiyasu, H., & Koeffler, H.P. (1995). Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood, 86*(3), 841-854.

Vagero, D., & Olin, R. (1983). Incidence of cancer in the electronic industry: using the new Swedish cancer environment registry as a screening instrument. *Br J Industrial Med, 40,* 188-192.

Van Dekken, H., Pizzolo, J.G., Reuter, V.E., & Melamed, M.R. (1990). Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet, 54,* 103-107.

Varga, E., & Tyldsley, W.R. (1991). Carcinoma arising in cyclosporine-induced gingival hyperplasia. *Br Dent J, 171,* 26-27.

Vaughn, T.L., Strader, C., Davis, S., & Daling, J.R. (1986). Formaldehyde and cancers of the pharynx, sinus and nasal cavity: Occupational exposures. *Int J Cancer, 38,* 667-683.

Vogelstein, B., & Kinzler, K.W. (1993). The multistep nature of cancer. *Trends Genet, 9,* 138-141.

Wang, L., Liu, T., Nishioka, M., Aguirre, R.L., Win, S.S., & Okada, N. (2006). Activation of ERK1/2 and cyclin D1 expression in oral tongue squamous cell carcinoma: relationship between clinicopathological appearances and cell proliferation. *Oral Oncol., 42,* 625-631.

Warnakulasuriya, S., Sutherland, G., & Scully, C. (2005). Tobacco, oral cancer and treatment of dependence. *Oral Oncol, 41*(3), 244-260.

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

Weinberg, R.A. (1989). Oncogenes, antioncogenes, and the molecular basis of multistep carcinogenesis. *Cancer Res, 49,* 3713-3721.

Williams, H.K. (2000). Molecular pathogenesis of oral squamous carcinoma. *J. Clin. Pathol. Mol.Pathol., 53,* 165-172.

Winn, D.M., Blot, W.J., & Joseph, K. (1991). Donald F. Austin, Raymond S. Greenberg, Susan Preston-Martin, Janet B. Schoenberg, and Joseph F. Fraumeni Jr. Mouthwash use and oral conditions in the risk of oral and pharyngeal cancer. *Cancer Res., 51,* 3044-3047.

Woolgar, J.A. (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol., 42,* 229-239.

World Health Organization (WHO) (2005). *The tobacco health toll.* Geneva.

Wright, N.A., & Alison, M. (1984). The biology of epithelial cell populations carcinogenesis. *Br7 Surg, 71,* 197-202.

Xiong, Y., Zhang, H., & Beach, D. (1992). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell*; 505-514.

Yamamoto, T., Kamata, N., Hiroko, K., Shoichi, S., Toshio, K., Kumao, T., et al. (1986). High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Res., 46,* 414-416.

Yoshida, K., Tanaka, T., Hirose, Y., Yamaguchi, F., Kohno, H., Toida, M., et al. (2005). Dietary garcinol inhibits 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rat. *Cancer Letters, 221*(1), 29-39.

Zain, R.B. (2001). Cultural and dietary risk factors of oral cancer and precancer – a brief overview. *Oral Oncol, 37*(3), 205-210.

Zanardi, A., Giorgetti, L., Oronza, A., Saverio, M., Paolo, M, Pier Giuseppe, P., et al. (2007). Immunocell-array for Molecular Dissection of Multiple Signaling Pathways in Mammalian Cells. *The American Society for Biochemistry and Molecular Biology, 6*(5), 939-947.

APPENDICES

APPENDIX I

FLUORESCENT IN SITU HYBRIDIZATION ASSAY PROCEDURE (VYSIS protocol)

a. Preparing specimen slides:

1. Cut 3µm thickness of paraffin sections using a microtomes.
2. Float the sections on the distilled water bath 45°C.
3. Mount a section on the silanized slide.
4. Air dried the slides.
5. Bake the slides overnight at 36°C.

b. Deparaffinizing slides:

6. Immerse slides in Xylene I for 10 minutes at ambient temperature.
7. Immerse slides in Xylene II for 10 minutes at ambient temperature.
8. Immerse slides in Xylene III for 10 minutes at ambient temperature.
9. Dehydrate slides in 100% Ethanol I for 5 minutes at ambient temperature.
10. Dehydrate slides in 100% Ethanol II for 5 minutes at ambient temperature.
11. The slides then dried in ambient temperature for 2-5 minutes.

c. Pretreating slides:

12. Immerse slides in 0.3 HCL for 20 minutes.
13. Immerse slides in autoclaved water for 3 minutes.
14. Immerse slides in Wash Buffer I for 3 minutes.
15. Immerse slides in Pretreating solution at 80°C for 30 minutes.
16. Immerse slides in autoclaved water for 1 minute.
17. Immerse slides in Wash Buffer I for 5 minutes.
18. Immerse slides in Wash Buffer II for 5 minutes.
19. Blot excess Wash Buffer on the paper towel.

d. Enzyme Treatment:

20. Immerse slide in Protease solution (pH=2) at 37°C water bath for 80 minutes.
21. Immerse slides in Wash Buffer I for 5 minutes.
22. Immerse slides in Wash Buffer II for 5 minutes.
23. Dried slides at ambient temperature for 2-5 minutes.
24. Rehydrate slides in 70% Ethanol I for 2 minutes.
25. Rehydrate slides in 85% Ethanol II for 2 minutes.
26. Rehydrate slides in 100% Ethanol III for 2 minutes.

e. Preparing for denaturation:

1. Add the following, for each target area, to a micro centrifuge tube at room temperature.

   7µL LSI/WCP hybridization buffer.

   1 µL probe

   2 µL purified water

2. Vortex and centrifuge tube for 3 seconds.

f. Hybridization step:

27. Mark hybridization area with a diamond tipped scribe on the specimen slide.
28. Apply 10 µL of probe mixture to one target area.
29. Apply cover slip immediately.
30. Cover slip with rubber cement.
31. Place the slides in pre-warmed humidified box overnight for 12-16 hrs in 37°C incubator.

g. Post hybridization wash:

32. Preparing couplin jar with 0.4X SSC and place into a 37°C water bath for a least 30 minutes.
33. Prepare second coupling jar with 2X SSC/0.1% NP-40 at ambient temperature.
34. Take out the humidified box from the incubator, when the hybridization time is completed.
35. Remove the cover slip from each slide.
36. Immerse 4 slides each time in 0.4X SSC; agitate the slide for 1-3 seconds.
37. Leave the slides in the coupling jar for 2 minutes in 37°C water bath.
38. Immerse slides in 2X SSC in 0.1% NP-40 at ambient temperature for 2 minutes. Agitate the slides for 1-3 seconds.
39. Air dry slides in darkness at room temperature for 1 hour.

h. Counterstain with DAPI:

1. Apply 10 µL DAPI counterstain to the target area.
2. Apply cover slip immediately.
3. Seal cover slip with rubber cement.
4. Examine the fluorescent signals under Fluorescence Microscope.
5. Keep slides in darkness at -20°C freezer.

APPENDIX 2

HAEMATOXYLIN AND EOSIN STAINING METHOD

Sections were placed in different solutions as below:

1. Dewaxing sections in 2 changes of xylene for 15 minutes and 3 minutes.
2. Hydrating sections with decreasing grades of ethanol (100% - 2 minutes; 95% - 2 minutes and 70% - 2 minutes).
3. Washing sections with running tap water for 3 minutes.
4. Nuclear staining with Harris Haematoxyllin for 3 minutes.
5. Differentiating staining with acid alcohol for 2 dips.
6. Washing sections with running tap water for 3 minutes.
7. Blueing the nuclear staining with potassium acetate for 2 minutes.
8. Washing sections with running tap water for 3 minutes.
9. Hydrating with 80% ethanol for 3 dips.
10. Cytoplasmic staining with eosin for 4 minutes.
11. Dehydrating sections with increasing grades of alcohol (2 changes of ethanol 95% - 1 minute; 2 changes of ethanol 100% - 1 minute).
12. Clearing sections with xylene (3 changes of 1 minute).
13. Mounting in dibutyl phthalate in xylene (DPX).

APPENDIX 3

DEFINTION of TNM STAGING (ADOPTED FROM AJCC)

Primary Tumor (T)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor 2cm or less in greatest dimension
T2	Tumor more than 2cm but not more than 4cm in greatest dimension
T3	Tumor more than 4cm in greatest dimension
T4 (lip)	Tumor invades adjacent structures (e.g. through cortical bone, inferior alveolar nerve, floor of mouth and skin of face)
T4 (oral cavity)	Tumor invades adjacent structures (e.g. through cortical bone, into deep [extrinsic] muscle of tongue, maxillary sinus, skin, superficial erosion a lone of bone/tooth socket by gingival primary is not the sufficient to classify as T4)

Regional Lymph Nodes (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension

Distant Metastasis (M)

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

STAGING GROUPING

StageGroup	T Stage	N Stage	M Stage
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4	N0	M0
	T4	N1	M0
	Any T	N2	M0
IVB	Any T	N3	M0
IVC	Any T	Any N	M1

APPENDIX 4

CLINICOPATHOLOGICAL FEATURES OF OSCC OF BUCCAL MUCOSA AND TONGUE

Registeration Number:

Name:

Age:

Gender: ☐ Male ☐ Female

Race: ☐ Malay ☐ Chinese

☐ Indian

Tumour Site: ☐ Tongue ☐ Buccal Mucosa

Tumour Depth:

Histological Grading:

☐ Well-differentiated ☐ Moderately differentiated

☐ Poorly differentiated

Pathological tumor size (T): ☐ T1 ☐ T2 ☐ T3 ☐ T4

Nodal status: ☐ N0 ☐ N1 ☐ N2 ☐ N3

Pattern of invasion: ☐ Cohesive ☐ Non-Cohesive

Pathological tumor grading: ☐ Stage I ☐ Stage II

☐ Stage III ☐ Stage IV

Date of diagnosis:

Date of treatment:

Cyclin D1 amplification: ☐ Positive ☐ Negative