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.