CHAPTER 1: INTRODUCTION

1.1. Back ground:

Approximately 3% of malignant tumours originate in the oral cavity and majority of them are squamous cell carcinoma (SSC) (Epstien et al., 2002). It has been suggested previously that almost 95% of intra-oral cancers are related to oral squamous cell carcinoma (OSCC) (Sugerman and Savage, 1999). OSCC is the sixth most common cancer worldwide and more than 300,000 new cases are diagnosed every year (Parkin et al., 1988). In most western countries, especially United Kingdom (UK) and United States of America (US), OSCC accounts for only 2% of malignancies. However, in India and other South East Asian countries, it remains as one of the most common cancers, where more than 40% of the cases account for malignant tumour (Paterson et al., 1996). Retrospective analysis of biopsy cases over a period of 29 years from Institute of Medical Research (IMR), (Ng and Siar, 1997) and series of biopsies cases diagnosed at the Faculty of Dentistry, University of Malaya (Siar et al., 1990) have also proved that OSCC forms the most commonly encountered malignant tumour of the oral cavity.

In Malaysian population, the prevalence of oral cancer has been found to be highest among the Indian race (Ng et al., 1985; Siar et al., 1990; Ng and Siar, 1997). In a population based study conducted in 1993/1994, it was observed that the indigenous people of Sabah and Sarawak also have high occurrence of oral precancerous lesions (Zain et al., 1997). This high prevalence or incidence of OSCC among these ethnic groups may be attributed due to the single most important habit, the betel-quid chewing which is still being widely practiced (Hashim, 1991).

The primary OSCC occurrence site includes buccal mucosa, tongue, alveolus, palate, lip and floor of the mouth. In Malaysia, tongue is the third commonest OSCC after buccal mucosa followed by mandibular gingival and dental alveolus as diagnosed by the Department of Oral Pathology, Oral Medicine and Periodontology, Faculty of Dentistry, University of Malaya (Siar et al., 1990).

OSCC is considered as an aggressive neoplasm. Despite the improved diagnosis and therapeutic aids, the survival rates for oral squamous cell carcinoma (OSSC) have been unchanged for decades (Baretton et al., 1995). It was observed that half of the patient diagnosed with OSCC would die within 2 to 3 years of diagnosis (Macfarlane et al., 1996; Bankfalvi and Piffko, 2000). At present, prediction of prognosis for patients with OSSC is mainly based on the TNM classification. However, the TNM classification does not fully predict the clinical course or reflect the biological properties of the tumours (Baily, 1991). In addition, oral cancer was definitively diagnosed and TNM classification established once after the cases have become locally advanced (Vokes et al., 1993). Any system that could possibly intervene prior to the advancement stages of cancer may be able improve the treatment results. Therefore, in addition to TNM system, there is a critical need for another/adjunct tool that can provide a better understanding of the course of the disease and also have the ability in early detection of the lesion/s. Previously, Silverman (1988) have reported that an early detection of small lesions have significantly improved the prognosis of patients.

Most physicians believed that negative or positive margins of tumours are essential in determining prognosis of patients with oral and pharyngeal cancer. A study by Kwok et al (2010) showed that patients with negative margins and those receiving repeated resections resulted in improved patient survival rate. Clonality of the cells which are

highly aneuploid was predicted by the aneuploidy hypothesis. In this hypothesis, Li et al (1999) suggest that the tumour antigen and possibly the other added genes generated aneuploidy which initiated karyotype evolution and ‘‘after 60 population doublings’’ would eventually generate clones of tumourigenic cells.

In recent years, cytometrically determined nuclear DNA content of tumour cells have been suggested as an important tool for indentifying the biological behavior of cancers. Previous studies have shown that the DNA-ploidy analysis is of prognostic importance in some human malignancies such as carcinomas of the ovary (Bresica et al., 1990), prostate (Merkel and Mcguire, 1990; Badalament et al., 1991), urinary bladder (Al-Abadi and Nagel. 1992; Norming et al., 1992), and malignant melanomas (Sorensen et al., 1991; Cohen et al., 1992). Some investigations have been published concerning the importance of DNA ploidy for prognosis in OSCC (Feichter et al., 1987; Tytor et al., 1989; Beltrami et al., 1992). In these studies different cytometric methods have been applied with different definitions of ploidy status and they used a wide variety of samples; fresh/frozen, paraffin embedded tumour tissue of biopsy and or resected specimens. This has resulted in the rates of aneuploidy to be varied greatly between 30% (Farrar et al., 1989) and 76% (Feichter et al., 1987). It was proposed that this wide variation is more likely to be due to the heterogeneity within lesions or sample variations rather than due to sample processing, varying diagnostic criteria for ploidy level and differences between the flow cytometry and cytophotometry analysis (Diwakar et al., 2005).

1.2. Rational of choosing DNA ploidy:

Despite its limitations as mentioned above, the analysis of DNA content of OSCC provides suitable prognostic information’s (Kaplan et al 1986), and is considered as one

of the most useful predictors of prognosis in head and neck tumours (Bundgaard et al., 1992). This is supplemented by the fact that chromosomal instability is now considered fundamental to the malignant phenotype and is proposed as a cause rather than the result of malignant transformation (Nigg, 2003; Rajagopalan et al., 2003). Rationally, the TNM system has been used as one of the prognostic indicators to evaluate the malignant severity of OSCC. However, the TNM system also have its shortcomings and thus, there is the need to look for an additional/adjunct tool to the TNM system. Though a number of studies have already proven the usefulness of using DNA ploidy as a prognostic indicator, but still there requires a necessity for further studies to support the previous studies.

1.3. Aim of study:

To explore the DNA ploidy status in OSCC and its association with its margins and sociodemographic-clinicopathologic characteristics. It is also the aim of this study is to investigate the association of the DNA ploidy status of the surgical margins and its pathologic types (clear and close margins)

1.4. Specific objectives of the study are:

1. To determine the prevalence of DNA ploidy in OSCC.
2. To investigate the association between DNA ploidy status of OSCC with its tumour surgical margins and the pathologic types of surgical margins.
3. To investigate the association between DNA ploidy status of surgical margins and the pathologic types of surgical margins.
4. To investigate the association between DNA ploidy status of OSCC and the selected clinico-pathological parameters.

1.5. Null hypothesis

1. There is no significant association between the DNA ploidy status of tumour and its surgical margins
2. There is no significant association between the DNA ploidy status of OSCC and the pathological types of surgical margins.
3. There is no association between DNA ploidy status of the surgical margins and the pathologic types of surgical margins.
4. There is no association between the DNA ploidy status of OSCC and the selected socio-demographic and clinico-pathological parameters i.e.:
    a. No association between ploidy status with age, gender, ethnicity and risk habits (smoking, betel quid chewing and drinking alcohol)
    b. No association between ploidy status with histo-pathological classification, pattern of invasion, pTNM staging, tumour site, tumour size, lymph node metastasis and depth of invasion.

CHAPTER 2: LITRATURE REVIEW

Oral cancers are defined as neoplasm involving the oral cavity. They cover a range of tumours that develop at different sites, including lip, tongue, gingiva, alveolus, buccal mucosa, floor of the mouth and oropharynx.

Oral squamous cell carcinoma (OSCC) is the most common histo-pathological type of oral cancer, accounting for approximately 91% of all oral malignancies (Silverberg et al., 1995) and its frequency is directly related to alcohol intake and smoking (Soder et al., 1995). The basal cell of oral epithelium has higher rate of mitotic activity than other parts of the human body. Of note, any disturbance in the quality or quantity of cell-regulating proteins can induce neoplastic growth in this location (Sapp et al., 1997). Although the advancement in surgery and radiotherapy have lowered the number of OSCC treatments, but their recurrence at the local site, or in the lymph nodes of the neck still prevails, and patients may develop the chances of getting second cancer or distant metastasis. This has ultimately reduced the overall survival to five-years. The available evidence strongly suggests, the failure in treatment in these patients are because of small numbers of cancerous cells that remains in the body after treatment, which are usually undetected by the current diagnostic techniques and thus those precancerous mucosa that remains undetected undergo malignant transformation and produces a new tumour. The prognoses of these SCC patients are generally poor mainly due to the late diagnosis (Silverman, 1988). Therefore there is great interest in identifying specific gene alterations that are potentially useful for the prevention and early diagnosis of SCC.

2.1. Epidemiology

According to Johnson (2001), when cancers of the mouth and pharynx are combined for

both genders, they rank sixth overall in the world, after lung, stomach, breast, colon and rectum, and cervix (plus corpus uteri). Throughout the world, malignant neoplasms of the mouth and pharynx rank as the fifth most common cancer in men and ranks seventh in women (Johnson et al., 1999). Further reports suggest that mouth and pharynx is the third most common site for occurrence of oral cancers among males in developing countries and fourth among females (Johnson, 2001).

Worldwide, the incidence of oral cancer varies enormously. High rates of oral cancer occurrence are reported in Asian countries like India and Sri Lanka and also in parts of France, Central and Eastern Europe and South America, where oral cancer is the commonest form of malignant tumours (40%) (Craig and Johnson, 1998; Johnson et al., 1999; Johnson, 2001). The incidence of oral cancer is highest among men in Northern France (49.4/100,000 men), and some areas of Eastern Europe and Latin America (Reichart, 2001). The variation in the incidence of oral cancers of head and neck regions are mostly related to the relative distribution of the major risk factors like tobacco or betel quid chewing, cigarette smoking and alcohol consumption (Sankaranarayanan et al., 1998).

The incidence of oral cancer is relatively low in most western countries (Paterson et al., 1996; Sugerman and Savage, 1999). The overall incidence rate for oral cancer in the United Kingdom is approximately 3.4 per 100,000 populations per year (Johnson and Warnakulasuriya, 1993). In many parts of India, the incidence rates have exceeded 6.0 per 100,000 per year and in South India a rate of 10.8 per 100,000 per year between 1991-1992 was reported (Moore et al., 2000).

In Malaysia, oral cancer has become a public health problem. Since 1976, the Division of Stomatology, Institute for Medical Research, Kuala Lumpur, Malaysia, have been

known to diagnose around 150-200 cases of oral cancer and precancers annually. The numbers of new cases reported are probably 1.5-2 times higher, since there are also other hospitals and laboratories that manage or report the data of oral cancer patients in Malaysia (Dental Services Division, Ministry of Health, Malaysia, 1997). In Peninsular Malaysia, about 60% of cases of oral cancers are observed among Indian populations even though they comprise about only 10% of the total population of the country. Great majority of the patients seeks for curative therapies only at the advanced stages of cancer (TNM stage III or IV). Most of the cases reported are related to betel quid chewing and they constitute the main high-risk group. In addition, communities in other parts of Sabah and Sarawak that indulge in these habits are also considered as a high-risk group (Dental Services Division, Ministry of Health, Malaysia, 1997).

Ng and Siar (1997) conducted a retrospective analysis of 29 years biopsy records of the Division of Stomatology, Institute for Medical Research, Kuala Lumpur and preliminary oral cancer surveys among various states of Malaysia. Their study confirmed that OSCC (90.8%) was the most commonly encountered malignancy in the oral cavity in Malaysian population and well-differentiated SCC was the most commonly encountered histological variant (Ng and Siar, 1997).

The general observation was that SCC among the Indian race showed a female predilection for all the histological variants, while an overall male predominance was observed in the Chinese ethnic group. A slight female predilection was observed among the Malay race. The first incidence data in Malaysia was reported by Hirayama (1966), whose study has shown that the incidence of oral cancer in the year 1963 was 3.1 per 100,000 populations. In this report, he also found that the incidence was highest among the Indian ethnic group and it varied among the individual states, with the highest rate

reported in the state of Selangor (8.2 per 100,000). After verifying the reports of Malaysian National Cancer Registry (MNCR) by the year 2001, Lim et al. (2002) have revealed that the incidence of oral cancer in Peninsular Malaysia to be around 1.6 cases per 100,000. They further revealed that the incidence of female to be 1.7 per 100,000 which was actually higher than the males (1.5 per 100,000). A study conducted by Mukhriz et al. (2003) on oral cancer incidence among 4 states of Malaysia, have showed that the age specific incidence of oral cancer (incidence rate adjusted to world population) was highest in the state of Negeri Sembilan (1.6 per 100,000) compared to the other states such of Perak (0.74 per 100,000), Sarawak (0.66 per 100,000) and Terengganu (0.48 per 100,000).

Within Peninsular Malaysia, mouth cancer ranked 21st among cancer in males and 16th in females. The incidence was highest among Indians. The age standardized incidence rates (ASR) of the Indian males in Malaysia (9.5 per 100,000) was lower than the ASR of Indian males in Trivandrum (10.8 per 100,000) and males in France (12.4 per 100,000) (Lim et al., 2002). However, the ASR for Malaysian Indian females (19.8 per 100,000) was found to be markedly higher than the ASR for females in the Indian subcontinent (Bangalore 8.9). Overall, when compared to all other cancers, mouth cancer was found to be the third most common cancer in Indian females and the sixth most common in males (Lim et al., 2002).

2.1.1. Age distribution

Oral squamous cell carcinoma is regarded as a disease of the elderly people and it has been suggested that the prevalence of oral cancer appears to increase with the age (Johnson, 1991). In the West, 98% of the oral cancer cases were over 40 years of age (Cawson and Odell, 1998). In Malaysia, the demographic analysis showed a late

adulthood onset with 71% of the cases occurring between 50 and 70 years of age (Ng and Siar, 1997). However, an increase number of cases have been reported among younger males in recent years (Hindle et al., 1996; Myers et al., 2000).

2.1.2. Gender distribution

The overall male to female ratio for OSCC will differ according to the sites and ethnicity. It was reported that for the OSCC of the tongue, the male to female ratio was not more than 1.7 to 1 and for the floor of the mouth, the male to female ratio was 2.5 to 1 (Cawson et al., 2001). In Malaysia, the retrospective analysis of the 29 years biopsy records of the Institute of Medical Research for oral cancer showed an overall male predominance with male to female ratio of 2:1 when all ethnic groups were considered. However, for well the Indian male to female ratio was 1:1.5. , The ratio of male to female incidence was 1:1.2 in Malaysian populations, when compared to other countries such as Singapore (1:0.97), United Kingdom, Norway (1:0.94) and Hong Kong (1:0.76) (Lim et al., 2002). There is variation of cancer incidence rate between the different ethnic groups. The crude incidence rate for cancers in Malay male and female was 60.6 and 79 per 100,000 populations, respectively; for Chinese male and females it was 169.2 and 217.7 per 100,000 populations, respectively; while for the Indian male and females, the incidence rate was 85.7 and 147.2 per 100,000 populations, respectively (Lim and Halimah, 2004).

2.1.3. Site distribution

Reports from western countries suggest that Oral cancers (excluding lip cancer) are most commonly affected in the lateral border of the tongue and the floor of the mouth. These regions are followed by the buccal mucosa, mandibular alveolus, retromolar region and soft palate with the hard palate and the dorsum of the tongue. However, all

these at lowest risk (Johnson, 1991). In Malaysia, the most commonly affected site is the buccal mucosa and gum (Lim and Halimah, 2004).

2.1.4. Ethnicity

The distributions of oral cancer among ethnic groups are strongly affected by the cultural and dietary habits in different geographical regions (Zain, 2001). In Malaysia, a nationwide survey in 1993 have shown that the prevalence of oral mucosal lesion in particular oral cancer and precancer showed variation among different ethnic groups, which may partly be contributed due to their differing diet and oral habits. The ethnic variations of certain lesions are evidenced in the nationwide survey conducted in Malaysia during the year, 1993/1994. Findings from this survey suggested that oral cancer and precancer were highest in the Indians, followed by the indigenous people of Sabah and Sarawak with the lowest prevalence in the Chinese (Zain et al., 1997).

2.1.5. Mortality

Oral cancer is a highly lethal disease with a range of 5-years survival rate of approximately 30-40% (Johnson, 1991). Within mouth, factors that influence the clinical outcome are size of the lesions at the time of diagnosis, degree of differentiation, the pattern of invasion and the proximity of carcinoma resection margins, lymph node status and presence of extra nodal spreads (Helliwell and Woolgar, 2000). High mortality rate of oral cancer can be attributed to the fact that most of the oral cancer cases that come to notice are at an advanced stage, where many of patients might have delayed seeking their treatments (Khoo et al., 1998) or might have refused the treatments previously (Gupta et al., 1987).

2.2. Aetiology of oral cancer

A large number of agents such as chemical carcinogens, radiant energy and oncogenic

microbes mainly viruses can cause genetic damage and have the ability to induce neoplastic transformation of cells. Causative or aetiologic agents for cancer are those for which laboratory or epidemiological evidence is available to support a carcinogenic potential (Johnson and Warnakulasuriya, 1993).

2.2.1. Tobacco smoking, betel quid chewing and alcohol

(a) Tobacco Smoking

Tobacco smoking is one of the most important risk factors for oral diseases including oral cancer (Johnson and Warnakulasuriya, 1993; Winn, 2001).Tobacco smoke has been identified as human carcinogen by the International Agency of Research on Cancer (IARC, 2002) with the target organ being the oral cavity, pharynx, larynx, oesophagus and lung. Tobacco smoking in all its forms constitutes a definite risk in the development of oral cancer (Johnson, 1991). Within the oral cavity, tobacco smoking was strongly associated with cancer in the soft palate (Bofetta et al., 1992) and the retromolar area (Jovanovic et al., 1993). A population-based case-control study have shown that risk of occurrence of oral cancer in cigarette smokers are two to five times more than that of non-smokers (Blot et al., 1988). Tobacco smoke has a direct carcinogenic effect on the epithelial cells of the oral mucous membrane. It has been well demonstrated that there is a dose-response relationship between the usage and the risk of development of oral cancer (Binnie et al., 1983; Johnson et al., 2000).

(b) Betel quid chewing

A variety of betel quid chewing habits are widespread in different parts of the world and this has led to considerable confusion as to whether or not investigators are describing the same habits in their studies (Zain et al., 1999). Therefore, in order to bring about some uniformity in the reporting of betel quid and tobacco chewing habits, a workshop

which was held in Kuala Lumpur, Malaysia during November 25-27, 1996 have given a definition. According to the recommendation from this conference, quid can be defined as a substance or mixture of substances placed in the mouth or chewed which remains in contact with the mucosa. These substances or mixtures may usually contain one or both of the two basic ingredients, tobacco and/or areca nut in raw or in any manufactured or processed form. The term betel quid was further defined by Zain et al. (1999) as specific variety of quid which indicates any type of mixture or quid that includes betel leaf.

Betel quid chewing consisting of mainly areca nut with betel leaf and other ingredients is common practice among Taiwanese and has been recognized as one of the most important aetiological factor for carcinogenesis (Ko et al., 1992; Lu et al., 1993; Ko et al., 1995; Chang et al., 2001). Chewing betel quid containing immature areca nut fruit seemed to carry a higher risk for developing oral cancer as compared to quid that included betel leaf without areca nut (Ko et al., 1995). The study further done using buccal carcinoma cell line which was defective in its ability to undergo differentiation have proved that extract toxicity could occur independently from the responses. Finally, the genotoxicity of the salivary tested using areca-nut-specific carcinogen 3-(N-nitrosomethyl-amino) propionaldehyde, have demonstrated the formation of DNA protein cross-links and DNA single-strand breaks in normal buccal epithelial cells (Sundqvist and Grafstrom, 1992).

The relationship between oral cancer and betel quid chewing in Malaysia has been well documented in many studies (Hirayama, 1966; Ramanathan et al., 1973; Ng et al., 1985; Hashim, 1991; Zain et al., 1999). The quid chewing habit appears to be a dying habit among younger Malaysians and urbanites. However, this habit is still being widely practiced among Indians working in plantations in remote urban centres, the indigenous

people in Sabah and Sarawak and some elderly Malays living in rural villages (Zain et al., 1999). The main quid ingredients used by the Malaysians are areca nut (dried and fresh), betel leaves and slaked lime (Zain et al., 1999). Tobacco is added to the quid mixtures especially among the Indians and indigenous people of Sabah and Sarawak (Ramanathan and Lakshimi, 1976; Rahman et al., 1999). The Malay quid chewers won't mostly include tobacco in their quid (Raman et al., 1999). Hashim (1991) has also shown that the betel quid had to be continued for at least a minimum of eight years before any evidence of mucosal changes could be detected.

(c) Consumption of alcoholic beverages

Excess consumption of any type of alcohol (spirits, beer and wine) raises the risk for developing oral cancer (Blot, 1992; Johnson and Warnakulauuriya, 1993; Fioretti et al., 1999; Franceschi et al., 1999). However, controversies exist as to which beverages carry the greatest risk. Kabat and Wynder (1989) have shown that beer and spirits have similar effects but Leclerc et al (1987) have found that wine drinking have more potential to be a causative for oral cancer. Mashberg et al. (1993) have claimed that mixed drinkers are at higher risk of oral cancer than drinkers of only one beverage type.

Oral cancer shows a strong, dose-dependent association with alcohol intake (Blot et al., 1988; Schildt et al., 1998; Franceschi et al., 2000), but is apparently unaffected by the duration of alcohol consumption as showed by Franceschi et al. (2000) where cessation of alcohol drinking did not give any clear favourable effects. Rising alcohol use has been shown to be related to the increase in oral cancer in the Western world have been directly related to the rising level of alcohol consumption (Hindle et al., 1996). Alcohol drinking is said to be strongly associated with the cancer of the tongue and the floor of the mouth (Boffeta et al., 1992; de Boer et al., 1997).

2.2.2. Viral, Fungal and Bacterial infection

Human papillomavirus (HPV) appears to be a significant independent risk factor for OSCC. HPV infection is associated with an increased risk (3 to 6 times) of OSCC independent of exposure to tobacco or alcohol. The relative risk of HPV and OSCC is equal to or exceed the risk associated with tobacco and alcohol consumption (Smith et al., 1998; Miller and Johnon, 2001). Human herpesvirus (HHV) especially HHV-6 and herpes simplex virus has shown to be linked with OSCC (Flaitz and Hicks, 1998). Another strain, Epstein-Barr virus (EBV) has been show to be more prevalent in OSCC than in normal mucosa, but the role of EBV in OSCC is still unclear (Sand et al., 2002).

Oral candidiasis is an important opportunistic infection especially in immuno-compromised patients like the human immunodeficiency virus (HIV) (Reichart, 2001). Patients with oral epithelial dysplasia or OSCC have recorded a higher number of yeast in their oral cavity than patients without any evidence of epithelial dysplasia or neoplasia histopathologically (McCullough et al., 2002). The surfaces of oral cancer are often invaded by yeast with Candida albicans being the dominant species (Krogh, 1990; Nagy et al., 1998). Syphilis infection has been associated with oral cancer especially the carcinoma of the 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 usually an unusual cancer site (Binnie et al., 1983). A study carried out between 1936 and 1968, reported that there was only 6.1% of the tongue carcinoma that were positive of syphilis (Meyer and Abbey, 1970). In a study to further elucidate the relationship of syphilis to cancer, Michalek et al. (1994), showed that there was an increase in cancer surveillance among people with syphilis though no conclusions can be reached concerning the causality.

2.2.3. Diet and nutrition

Differing diet, which leads to deficiency, may partly contribute to the prevalence of oral cancer and precancer (Zain et al., 1999). Nutritional deficiency produces atrophy of oral and other mucous membranes and may render them more susceptible to local carcinogens (Johnson, 1991). Carotenoids and some vitamins such as vitamin A, C and E have been shown to give protective effect against some cancers (McLaughlin et al., 1988; De Stefani et al., 2000; Royack et al., 2000). Iron deficiency has been associated with oral cancer, which was classically expressed in Plummer-Vinson or Paterson-Kelly syndrome (Wynder et al., 1957). Dietary iron plays a vital role in maintaining the epithelial thickness (Ogden and Macluskey, 2000).

2.2.4. Occupation

Occupation as a risk factor has been studied to a lesser extent. Epidemiological evidence exists for an association between workers exposed to formaldehyde (Blair et al., 1986; Vaughn et al., 1986), and other manual workers such as printers (Lloyd et al., 1977; Dubrow et al., 1984), electronics workers (Vagero and Olin 1983), and textile workers (Blot and Fraumeni, 1977) have increased risk of oral cancers.

2.2.5. Immune defence and Genetic factors

It is possible that immunosuppresion, either by drug or by HIV infection may play a role in imparting a high risk status for the development of oral carcinoma (Johnson, 1991). Carcinoma of the lip has been reported to be associated with a number of kidney patients receiving immuno-suppressive medications (De Visscher et al., 1997). In another study, it was observed that oral leukoplakia had undergone rapid progression to carcinoma in an immuno-suppressed liver transplanted patient (Hernandez et al., 2003). Oral cancer tends to aggregate in families, revealing the genetic traits also a causative

factor. A study from Kerala state, India has revealed that a familial aggregation, mostly site-specific, with an autosomal dominant mode of inheritance in 0.94% of total oral cancers (Ankathil et al., 1996). In this study, Ankathil et al. (1996) also observed that a family history of oral cancer was associated mostly with an early onset of the disease.

2.2.6. Mouth rinse

Regular use of mouthwash has been associated with increased risk of oral cancer (Winn et al., 1991). In an interview with oral cancer patients from the general population of US, it was revealed that the risk of oral cancer was elevated by 40% among male and 60% among female mouthwash users (Winn et al., 1991). Proprietary mouthwashes are a source of ethanol, which may act locally in a manner similar to alcohol drinking (Johnson and Warnakulasuriya, 1993). Risks generally increased in proportion to frequency and duration of mouthwash usage (Winn et al., 1991) and were only apparent when the alcohol content of the mouthwash has exceeded 25 percent (Johnson, 2001).

Besides alcohol, sodium lauryl sulphate (SLS), an important component in many oral health products has been suggested to affect the structural integrity of oral mucosa (Healy et al., 2000). Though much has been debated, SLS has not been classified as a carcinogen (not even in group 4). However, a study has shown that the addition of triclosan and zinc to these oral products appeared to prevent the damaging effect on tissue permeability of SLS on oral mucosa (Healy et al., 2000).

2.2.7. Ultraviolet radiation

The ultraviolet radiation is an important aetiology for the SCC of the vermilion border of the lip for people who lives or works outdoors (Wurman et al., 1975; Johnson, 1991). The upper lip is infrequently involved because it is less directly exposed to the sun (Wurman et al., 1975). A study in California showed that the risk of lip cancer for

women is strongly related to lifetime solar radiation exposure (Pogoda and Preston-Martin, 1996). In doses equivalent to recreational sun exposure, UV radiation may also be immunosuppressive, which may increase the chance of malignantly transformed cells in escaping the immunological surveillance. These malignantly transformed cells may increase the opportunity for virus infection and malignantly transform the keratinocytes (Parrish, 1983).

2.3. Carcinogenesis

Cancer is caused by a series of genetic changes, each potentially leading to a clonal outgrowth of cancer cells with a selective growth advantage (Boyle et al., 1993). Cancer is a multistep process which involves initiation, promotion and tumour progression (Scully, 1992) and genetic damage may occur at any point in the initiation, promotion and progression of the disease (Macluskey & Ogden, 2000). Initiation involves mutational events in unknown genes may be caused by chemical carcinogens, radiation or viruses and is thought to be irreversible (Macluskey & Ogden, 2000). The latter stages of promotion involve further genetic alterations which lead to malignancy (Macluskey & Ogden, 2000). The expansion of a malignant clone eventually leads to local invasion and possible distant metastasis (Macluskey & Ogden, 2000).

2.4. Molecular biology of oral cancer

Models of tumour genesis involve multiple molecular events such as proto-oncogenes and tumour suppressor gene. The proto-oncogene whose function is to promote cell proliferation, carry out positive stimulations leading to continuous signalling which acts positively on cell growth (Seemayer and Cavenee, 1989; Scully, 1992), and in the other hand tumour-suppressor genes whose products inhibits the cell proliferation thus

become inactivated, which leads to unchecked nepolastic growth in the tumourigenesis (Weinberg, 1989; Stanbridge and Nowell, 1990; Vogelstein and Kinzler, 1993).

Van der Riet and co-workers (1994) has assembled the genetic events of SCC of the head and neck (HNSCC) and they investigated whether there are any involvements or association of the cytogenetic alterations, interaction with viral products, or radiation damage or chemical carcinogens with HNSCC. The importance of each alteration in the development and precise sequence of cell carcinoma of the head and neck has not been fully established.

Rationally, loss of heterozogosity on chromosome 3p, 9p, 11p, 13p and 17p reported in oral cancers suggests that there could be a possible pathway for the progression of oral carcinogenesis, which might involve an increased rate of errors during DNA replication and defective repair of DNA. The occurrence of multiple areas of allelic loss in several chromosomes, together with the sequential loss of several tumour suppressor genes (TSGs) during experimental carcinogenesis, is entirely consistent with the hypothesis that oral carcinogenesis involves multiple molecular steps (Califano et al., 1996; Todd et al., 1997).

2.5. Tumour cell cycle

Cancer is also considered as a cell cycle disease (Bartek et al., 1999). Defects in the cell cycle will alter the cell division cycle leading to an increased cell proliferation and may result in development of tumour. Such defect can either target components of the cell cycle apparatus itself, checkpoint mechanism, or even the target elements of the upstream signalling cascades. The net result is deregulation of the cell cycle division leading to cancer-prone cellular environment. Cell cycle deregulation is an essential step in the process of multistep tumourogenesis (Bartek et al., 1999).

Cell cycle is defined as the interval between the completion of mitosis in a cell and the completion of mitosis by one or both of its daughter cells (Goodger et al., 1997). The study of cell kinetics began in 1951 and discovered that DNA syntheses are occurred in discrete intervals in the cell cycle of mitosis. It has been concluded that there must be at least four distinct phases within the cell cycle (Jones et al., 1994).

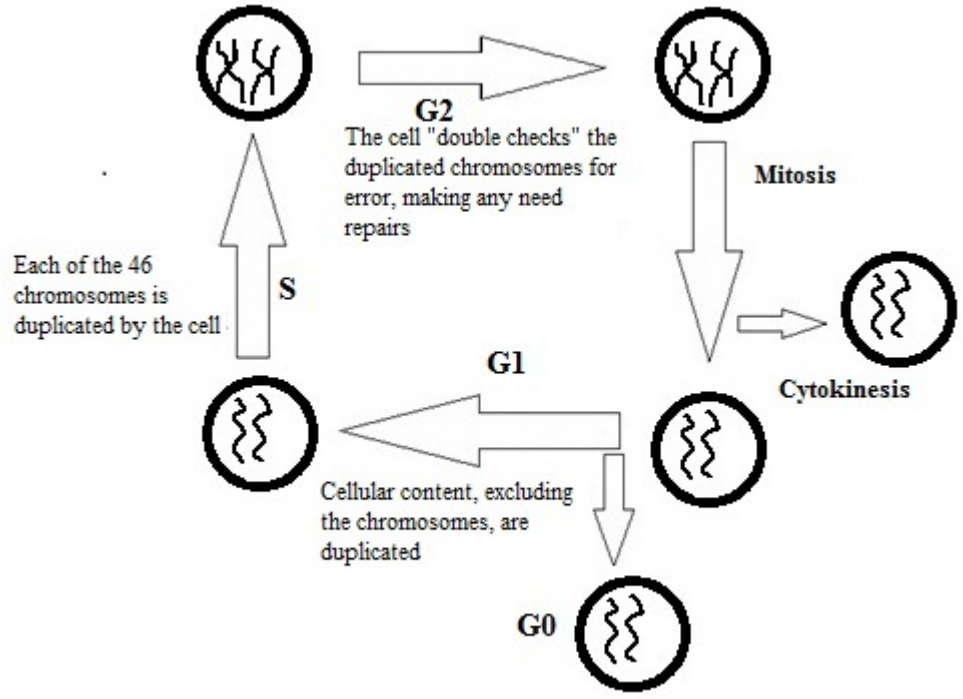


Figure 2.1: Diagrammatic representation of a cell cycle. The cell cycle growth consist of G1-phase (presynthetic), S-phase (DNA synthesis), G2-phase (premitotic) and M-phase (mitotic). Note: Stable cells are in G0 phase.

In the first phase, known as gap 1 (G1), the cell undergoes biochemical changes to prepare itself to enter into S-phase. DNA synthesis and doubling of the genome take place during the synthesis or S-phase. The S-phase is followed by a second preparatory phase known as the second gap phase (G2) which comes before the next mitosis. In M-phase, the replicated DNA is carefully condensed into compact chromosomes that are precisely segregated so that two daughter cells, each of which receives a full complement of the genetic material. Following mitosis, a proliferating cell directly re-

enters the G1-phase as it prepares for further replication. Other proliferating cell has an additional option, which is the entry into quiescent state known as G0 (Hall and Levison, 1990; Kastan and Skapek, 2001).

Cells in the resting phase (G0) are stimulated by growth factors produced by proto-oncogenes to enter the cell cycle. The cell cycle and cell growth are controlled by many factors including signals from other cells. Sending a signal is by producing a change in its cell surface or secreting a substance which can affect the target cell. The signalling substance (a hormone or other factor) must bind to a surface receptor or the target cell; this interaction then triggers a signal within the target cell resulting in a change in the growth of the cell. For example, epidermal growth factor (EGF) stimulates cell growth in the epithelium, while transforming growth factor (TGF-) inhibit growth. The ultimate consequence of this signalling is that the nucleus responded by the transcription of particular genes requires a passage through the cell cycle of the induction of differentiated characteristics (Scully, 1993).

Cells only undergo division in response to the correct growth stimulating signals and there are important checkpoints at various stages of the cell cycle where these controls or checkpoints must receive and transmit another signal to other component that regulate this process, if cell division is to occur (Partridge, 2000). There are cell cycle control proteins that govern each step in the cell cycle. Without them, the cell cannot divide. The first and most important functions of the controlling proteins are as ‘checkpoint’ (Goodger et al., 1997). There are at least 4 ‘checkpoints’ which are irreversible steps: G1, the restriction point (R), the point from which the cell is committed to mitosis, G1/S phase, G2/M and the metaphase /anaphase transitions (Goodger et al., 1997). Faults in this ‘checkpoint’ mechanism may allow genetically

abnormal cells to undergo division leading to the accumulation of genetic defects allowing tumour initiation and progression (Goodger et al., 1997).

The second function of the controlling proteins is the maintenance of the chronological order of the cell cycle, ensuring the strict alteration of M and S phase, thus preventing any loss of sequence or repetition (Goodger et al., 1997). Cellular proliferation follows an orderly progression through the cell cycle, which is controlled by protein complexes composed of cyclins and cyclin-dependent kinases (cdk) (Cordon-Cardo, 1995). During G1, a series of cyclin proteins are synthesized which activate cyclin-dependent kinases (cdk). These kinases activities are required for commitment of the cell to DNA synthesis (S phase) and for completion of cell division (Partridge, 2000).

Multiple cyclins have been isolated and characterized. Eight major classes of mammalian cyclins (termed A-H) have been described (Goodger et al., 1997). Cyclin A is synthesized during S phase. The appearance of cyclin A in the cell coincides with DNA synthesis. It is required for S phase and for passage from G2 into mitosis. Cyclin B mRNA peaks at the G2/M transition following a gradual rise through S phase and cyclin B protein appear in the cytoplasm in the late S phase. A and B are regarded as regulators of the transition to mitosis.

Cyclin C peaks in mid-G1, cyclin D2 appears in late G1 and cyclin E undergoes a sudden onset of transcription at mid to late G1. Cyclin G has been identified as a possible transcriptional target of p53, while cyclin H is found in G2. Multiple cdk molecules are being identified and their cyclin partner and patterns of cell cycle specificity distinguished. The complexes formed by cyclin D1 and cdk4 govern G1 progression, while cyclin E-cdk2 controls entry into S phase. Cyclin A-cdk2 units affect

their regulation through S phase, and cyclin B-cdc2 (cdk1) control entry into mitosis (Goodger et al., 1997).

Cyclin/cdk activity is controlled by interactions between cdks or cyclin/cdk complexes and other proteins known as cyclin/cdk inhibitors (CKIs) (Kastan and Skapek, 2001). Two groups of CKIS have been identified; $p21^{WAFI/CIPI}$, $p27^{KIPI}$ and $p57^{KIP2}$ which appear to be universal inhibitors of cyclin/cdk activity and the second group which comprises of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ which specifically inhibit cyclin D-associated cdk4 and cdk6 (Kastan and Skapek, 2001).

2.6. Clinical presentation of oral cancer

Oral cancer may present itself clinically as either singly or as a mixture of presentations as follows (Zain et al., 2002):

As a white lesion: This lesion may develop as a white area but is indurate. The surface may be nodular or ulcerated. There may be fixation if the tissue occurs on a movable part of the mucosa.

As a red lesion: This lesion may develop as a red area but there is induration where the tissue feels firm and thickened throughout the lesion or at the margins if ulcerated.

As an ulcerated lesion: This lesion is ulcerated with indurations at the ulcer margins. The ulcer may have a raised, rolled border and may develop in a white area. It should be distinguished from a large solitary major aphthous ulcer, traumatic ulcer or infectious ulcer.

As an exophytic mucosal swelling: OSCC may appear as a fungating exophytic mass which could easily bleed at later stages of lesions. It may also appear as painless with a warty or white nodular surface as in verrucous carcinoma.

As to which form of lesion, an OSCC present itself is partly influenced by the habits practiced in different geographical locations (Paterson et al., 1996). In Western countries, where tobacco smoking and alcohol consumption are the most widely practiced habits (Mashberg et al., 1993; Franceschi et al., 1999), have caused OSCC to be typically found near to the lateral border of the tongue, floor of the mouth and lingual aspect of the lower alveolus. These occurrences are possibly due to the pooling of carcinogens from both smoke and alcohol. These tumours are usually endophytic and may have the ability to deeply penetrate (Paterson et al., 1996). In countries where there is a widespread habit of betel quid and tobacco chewing, tumours are commonly developed in their buccal sulcus and the buccal mucosa (Paterson et al., 1996; Pindborg et al., 1997). These tumours are usually exophytic and may be large at the presentation (Paterson et al., 1996). Majority of the tumours of the buccal mucosa are located posteriorly (Pindborg et al., 1997). In Malaysia, occurrence of ulcerations or swellings, either separately or together is considered to be one of the main clinical signs of OSCC. The ulceration is normally crateriform in appearance while the swelling often took the form of a cauliflower like or fungating growth (Siar et al., 1990).

2.7. Histopathological features of oral cancer

Histopathological examinations are critical since it allows grading of neoplasm, which allows in predicting their aggressiveness. This would enable to establish proper prognosis for the patient or as a best indicator for the most effective treatment. This grading is generally based on the method originally described by Broders in the year 1920, which takes into account a subjective assessment of the degree of the keratinisation, cellular, nuclear pleomorphism and mitotic activities (Pindborg et al., 1997). The detailed description of the different category of OSCC is as below:

Grade I: Well-differentiated: Histological and cytological features closely resemble to those of the 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 mitoses or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal.

Grade II: Moderately-differentiated: This is a neoplasm with features intermediate between well-differentiated and poorly differentiated SCC. Compared with well-differentiated SCC, these have less keratinization and more nulear and cellular pleomorphism; there are more mitotic figures and some are abnormal in form. Intercellular bridges are less noticeable.

Grade III: Poorly-differentiated: Histologically and cytologically there is only slight resemblance with the normal stratified squamous epithelium of the oral mucosa. Keratinization is rarely present and intercellular bridges are extremely rare; mitotic activity is frequent and typical mitoses can readily be found; cellular and nuclear pleomorphism are obvious and multinucleated cells may be frequent.

Studies done by Melchiorri et al. (1996) and Baretton et al. (1995) have found that there was no correlation between the DNA ploidy parameters and histopathologic grading of tumour.

2.8. Clinical and Histological prognostic indicators of oral cancer

2.8.1. Tumour stage

TNM staging system is the most commonly applied classification used in clinical practice to assess the patient's prognosis in cancer (Macluskey and Odgen, 2000) and it

is claimed to be the simplest, cheapeast, relatively accurate, objective and universally accepted protocol (Piccirillo, 1995). The TNM system requires clinicians to describe the nature of the tumour extension by the T (extent of the primary tumour), N (the status of the regional lymph nodes involvement) and M (the presence or absence of distant metastasis) (Hermanek and Sobin, 1992). The primary purpose of clinical staging is to divide patients according to prognostically meaningful groups, similar disease severity and prognosis (Jones et al., 1993; Hall et al., 1999).

Pathological version of the TNM classification also exist, designated pTNM, where its criteria of evaluation consists of those of clinical TNM system supplement by the evidence found after excision and histopathological examination and is believed to be an important aspect in estimating prognosis and calculating the end results (Hermanek and Sobin, 1992).Many modification of the TNM system have been done previously (Jones et al., 1993; Synderman and Wagner, 1995; Hall et al., 1999). All of these systems have highlighted the importance of nodal involvement in assessing the prognosis and some have proved to be prognostically valid in predicting the survival (Jones et al., 1993; Hall et al., 1999). Unfortunately, inaccuracy still exists in measuring nodal status, as preoperative diagnosis of the lymph node metastasis by routine methods remains a big concern (Macluskey and Odgen, 2000).

Numerous investigations have shown that TNM staging is an important predictor of OSCC prognosis. According to the American Joint Committee on Cancer (AJCC, 1997), the 5-year relative survivals for SCC of the oral cavity by stages are as follows:

Stage I, 65 to 70%

Stage II, 50 to 55%

Stage III, 38 to 44%

Stage IV, 25 to 29%

Other studies have also shown that the 5-year survival varies between stages where the 5-year survival is 75%, 65.5%, 49%, and 30% for stage I, II, III, and IV cancers of the oral cavity, respectively (Werning. 2007). Werning et al. (2007) also reported that the 5-year disease-specific survivals for 227 patients with floor of mouth cancer at different stages were; stage I, 72.4%; stage II, 62.8%; stage III, 44.4%; and stage IV, 46.9%. The same investigators also reported the 5-year disease-specific survival in a retrospective case series of 279 patients in oral tongue cancer as; 75.8% for stage I; 63.5% for stage II; 38.5% stage III and 26.5% stage IV. These findings validate the utility of the AJCC staging system as a predictor of prognosis and survival (Werning, 2007).

Currently, the TNM classification system is often supplemented with the histopathological tumour grading (tumour differentiation) which helps in therapeutic decision-making (Roland et al., 1994; Bankfalvi and piffko, 2000). However, the present TNM system cannot always be accurate (Macluskey and Odgen, 2000). Though histopathological grading of OSCC may be meaningful (Welkoborsky et al., 1995), but it is not a factor to be solely depends upon to which treatment decision can be taken (Roland et al., 1992). This has lead to the study of finer detail within tumour tissue using immunohistochemical and other molecular-biological techniques to study various markers associated with changes in malignancy (Macluskey and Odgen, 2000).

Earlier researches on OSCC have found no relation or partial relation of the N (lymph node metastasis) or T (tumour size) with the ploidy level. Baretton et al. (1995) have found that ploidy status of the tumour cells showed no correlations with patients' age and sex or pathological TNM system (pTNM) stage and grading (WHO), however, the pT stage have proved to be a statistically significant indicator for prognosis of the 90

patients with known follow-up. In another study, Melchiorri et al. (1996) also found that none of the clinical parameters (age, tumour site, tumour size and lymph node status) was significantly associated with the DNA ploidy.

Struikmans et al. (1998) found a higher probability of loco-regional recurrent disease to be associated with T4 stage of head and neck SCC. There are limitations in studies conducted on TNM staging for predicting prognosis. Some of the known limitations are:

Uniformity: There should be homogeneity within each stage grouping so that survival rate is similar between members of the stage group.

Discrimination: There should be heterogeneity between each stage grouping so that survival rates differ (the survival rate in group III should be worse than that for those in group with stage II).

Predictive power: The prediction of cure for particular stage grouping should be high (a group of stage I patients should manifest similar prognoses over time).

Balance: A balanced distribution of patients into each stage grouping permits meaningful statistical comparisons between groups (Werning, 2007).

2.8.2. Tumour size and tumour thickness/depth of invasion

Tumour size, thickness and depth of invasion are considered to harbor prognostic value (Speight and Morgan, 1993; Kristensen et al., 1999). Size of the tumour is normally graded in the range of three sizes. Tumour which are <2 cm have a significantly better prognosis than other two size 2-4 cm and >4 cm size (Speight and Morgan, 1993). In fact, size 2-4 cm have better prognosis than >4 cm size, but not as better as size <2 cm.

Tumour depths of invasion have shown its importance in predicting cervical metastasis (Fukano et al., 1997). Depth of invasion of <5mm have significantly better prognosis

than invasion of >5mm (Speight and morgan, 1993). This 5 mm discerning point was also observed by Fukano et al. (1997) where the incidence of cervical metastasis was increased clearly when the depth of invasion was over 5 mm. Hence, elective neck surgery (surgery or radiation) is usually recommended to perform on tumours with depth of invasion more than 5 mm.

Tumour size and depth of invasion were highly correlated to each other. A study done by Kristensen et al. (1999) indicated that patients with small tumours less than 2 cm in diameter and larger tumours with a depth of invasion less than 1 cm were considered as a low risk group with a 5-year disease-free survival of 95%. Site of origin of the primary tumour are considered to be prognostically valuable in OSCC (Platz et al., 1983; Baatenburg de jong et al., 2001). For example; tumours of the lip have a better prognosis than that of the oral cavity, which in turn was better compared to oropharynx (Speight and morgan, 1993). Within oral cavity, more posteriorly located tumours have poorer prognosis (Cawson et al., 2001).

It was possible to predict the survival probabilities in a new patient with head and neck SCC. This was done based on historical results from a data-set (hospital-based data on site of tumour, tumour size, age at diagnosis and TNM staging) which was analysed with Cox-regression model. Study suggests that the critical thickness was highly site dependent, but 4 mm is a useful average size for OSCC. Actually thicker tumours are having a fourfold increased risk of nodal metastasis than thinner tumours (Ambrosch et al., 1995).

The TNM system takes only tumour diameter into consideration during tumour staging, excluding other size parameters such as area, volume and depth. However, many studies have confirmed that among all these parameters of tumour size measurements, tumour

depth seems to be the only independent prognostic factor with a consistently adverse effect on lymph node metastasis, local recurrence and survival rate in mobile tongue cancer (Bello et al., 2010).

The assessment of maximal tumour thickness has been so far applied only to tumours of the skin and the cervix, but rarely has been applied to other carcinomas including the oral cavity. Frierson and Cooper (1986) have found a correlation between microscopic thickness and prognosis in a series of lower lip SCC. They concluded that the cut-off level for prognostic assessment is of 6 mm: three quarters of the patients with at least 6 mm of invasion have observed to develop metastases (Frierson and Cooper, 1986).

Tumour thickness and depth of invasion are the two important prognostic factors in oral cancer, but they are not being utilized for the tumour staging in the American Joint Committee on Cancer (AJCC) classification scheme. Tumour thickness and depth of invasion are predictive of occult nodal metastasis in the clinically negative patient's neck with oral cavity cancer. The critical thickness that is associated with an increased risk of metastasis, however, remains ill-defined and may not be uniform throughout the oral cavity. Future investigative efforts to characterize the relationship between depth of invasion and occult metastatic disease are necessary. This could be facilitated by routinely measuring depth of invasion in a prospective manner and recording these findings on the form of tumour staging (Werning, 2007).

2.8.3. Lymph node status

Lymph node involvement has been shown to directly affect the prognosis of oral cancer (Yamamoto et al., 1984; Jones et al., 1994). Variables related to lymph node metastasis are presence, number and the location of the node/s and the nodal extra-capsular involvement. These factors have been regarded as significant prognostic factors for oral

cancer (Brennan et al., 1995; Kowalski et al., 2000). The level of lymph node involvement has been associated with prognosis, where higher levels of lymph node gave poor prognosis (Jones et al., 1994; Kowalski et al., 2000). Kowalski et al. (2000) have reported that the risks of recurrence and death were significantly higher (more than 2.5 times) for cases of lymph node involvement at levels 3, 4 or 5 in relation to cases with no metastatic lymph nodes. The level of lateral lymph node involvement was recognized as the most significant prognostic factor in oral cancer patients who underwent surgical treatment (Kowalski et al., 2000).

Spreading of lymph node metastases beyond the sentinel lymph nodes (SLNs) has been regarded as the most important prognostic factor (Mamelle et al., 1994; Jones et al., 1994). SLNs are radioactive stained lymph nodes that were visualized during intraoperative lymphatic mapping using a gamma probe: visualization of blue stained lymph identified blue SLNs (Shoaib et al., 2001). This procedure has improved the diagnosis of micrometastasis in the regional tumour-draining lymph nodes by providing a focused histopathological assessment of the selected lymph nodes which are most likely to harbor occult disease (Taback et al., 2002). SLN is an accurate reflector of the status of regional lymph nodes when found in patients with early tumour (Shoaib et al., 2001). Baretton et al. (1995) showed that aneuploid tumours turned out to have lymph node metastases more frequently than diploid OSCC.

The DNA content in primary tumours of the oral cavity and corresponding lymph node metastases have indicated that aneuploidy are likely to happened before dissemination of metastatic cells Melchiorri et al. (1996). Staibano et al. (1998) showed that the DNA aneuploidy in these tumours were closely associated with their metastatic potential. Huang et al. (2009) suggested the optimal cut-off point for tumour depth predictive of

cervical nodal metastasis of OSCC to be 4 mm. Rahima et al. (2004) showed that perineural invasion apparently correlates with higher probability of regional and distant metastases, higher depth of tumour invasion, lower differentiation and lower 5-year survival rates in OSCC. Rodolico et al. (2004) showed that perineural invasion correlates with the risk of nodal metastases in OSCC.

2.9. Surgical margin involvement and field cancerization of oral cancer

Cancer patients share the common high risk of developing a simultaneous or subsequent second primary cancer within the anatomical tract of aerodigestive epithelium, which is caused due to the consequence of field cancerization (Bankfalvi and Piffko, 2000). The hypothesis behind field cancerization is that there are carcinogens which could induce changes throughout the mucosa of the upper aerodigestive tract of the head and neck cancer patients (Califano et al., 1996) and it is the description of phenomenon by which an entire field of tissue are developed as malignant or premalignant in response to a carcinogen (Califano et al., 1996).

The mechanism behind field cancerization is unknown (Sugerman and Savage, 1999). Three hypotheses have been proposed by Ogden (1998) on field cancerization:

1. Field changes or molecular changes throughout the oral mucosa of oral cancer patients may predispose to the development of multiple primary cancers. A large region of the oral mucosa may be exposed to the aetiological agents which causes independent transformation of multiple epithelial cells at separate sites.
2. The aetiological agents may transform a single oral epithelial cell. The expanding clone of cancer cells may spread through the oral mucosa via local tissue spread, regional blood vessels, seeding via the saliva into mucosal erosion or seeding due to the trauma of surgery. This would give rise to geographically

distinct but genetically identical cancers. A tumour may have a paracrine effect on the adjacent oral mucosa.

3. Evaluation of the surgical margins should distinguish between the superficial margin (mucosal) and deep resection margins as deep involved margins are associated with recurrent tumours that may be difficult to detect at an early stage.

Involved margins are associated with a poor prognosis. Woolgar and Triantafyllou (2005) have found that only 11% of patients with involved margins were alive after 5 years compared to 78% with clear margins and 47% with close margins. Margins of 5 mm or more are considered clear, 1–4 mm as close and less than 1 mm as involved (Helliwell and Woolgar, 1998; Woolgar and Triantafyllou. 2005). A strong correlation has been demonstrated between the resection margin free of disease and higher survival rates with longer time until recurrence of disease (Al-Rajhi et al., 2000).

2.10. DNA content

In order to explain DNA content, it is necessary to know about the three terms used in relation to DNA content namely Chromosome, DNA and Gene:

A chromosome is a structure containing genetic material (DNA but RNA in some viruses) termed genetic elements. The genome is the total complement of genes in a cell or virus (Madigan and Martinko. 2006).

Chromosomes are formed during mitosis by condensation of the euchromatin and combination with heterochromatin. Each chromosome is formed by two chromatids that are joined together at a point called the centromer. The double nature of the chromosome is produced in the preceding synthetic (S) phase of the cell cycle, during which DNA is replicated in anticipation of the next mitotic division. With the exception

of the mature gametes, the egg and sperm, human cells contain 46 chromosomes organized as 23 homologous pairs (each chromosome in the pair has the same shape and size). Twenty-two pairs have identical chromosomes (each chromosome of the pair contains the same portion of the genome) are called autosomes. The twinty-third chromosomes are the sex chromosomes, designated X and Y. Females have two X chromosomes and males have one X and one Y chromosome (Ross et al., 2003).

The deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are macromolecules composed of monomers called nucleotides. The DNA carries the genetic blueprint for cell while RNA is the intermediary molecule that converts the blueprint into defined amino acid sequences into proteins. A nucleotide is composed of three components: a five-carbon sugar, either ribose (in RNA) or deoxyribose (in DNA), a nitrogen base, and a molecule of phosphate, PO_4^{3-}.

The functional unit of genetic information is the gene. All life forms contain genes. Genes are composed of deoxyribonucleic acid (DNA). The information in the gene is present as the sequence of bases-purine (adinine and guanine) and pyrimidines (thymine and cytosine) in the DNA (Madigan and Martinko. 2006).

Nuclear deoxyribonucleic acid (DNA) content is a prognostic factor in several tumors, and decisions regarding treatment have been made using this parameter (Rubio Bueno et al., 1998). Normal resting human cells have 46 chromosomes. During proliferation, the DNA content doubles. Cells that are replicating DNA (in S-phase of the cell cycle) will have an intermediate content of DNA. In malignancy, structural and/or chromosomal aberrations are common. Only when the net chromosome number is changed, can deviations in the DNA content from normal be observed, giving rise to DNA aneuploidy. Thus lack of abnormality in the DNA content does not exclude malignancy

or the presence of chromosome abnormalities. Ploidy was originally used to refer to the chromosome number. In cytometry, it is used to describe the overall DNA content. Diploid cells have a DNA content of normal cells although their chromosomes may be abnormal (Ormerod et al., 1998).

One of the earliest methods for assessing the prognosis of cancer is to measure DNA content in cancer cells, either by image analysis with smears stained with feulgen stain, or by flow cytometry in cell or nuclear suspensions. For a number of years, measurement of total DNA content within the cells has been applied mainly for the prognosis of solid tumours (Merkel and Mcguire. 1990; Robinson, 1992).

2.10.1. Measuring DNA Content

There are two types of measurement for DNA content:

Assessment of chromosome number: This is based on the abnormality in total chromosome number for the malignant cells. The frequency of these abnormalities generally increases with progression to higher grade tumours.

Measurement of DNA assessment in the S-phase fraction: Proportion of the cells with DNA content usually reflects the rate of cellular division within the tumour. A higher S-phase fraction correlates with greater tumour aggressiveness.

DNA content can be analyzed using following techniques:

1. Flow cytometry
2. Image cytometry

Flow cytometry is performed on disaggregated cells or nuclei in suspension. Image analysis microscopy is performed on cells in smears or tissue sections. Flow cytometry can be also carried out using formalin fixed paraffin-embedded tissues (Peistring et al.,

1990). For both techniques, the cells are first stained with dyes that bind quantitatively to DNA. For flow cytometry, fluorescent dyes are used. The fluorescent stained cells will then be analyzed using flow cytometry, which excites the dye with a laser beam and measures the fluorescence emitted from individual cells as they pass through detector. The fluorescence data are compiled in a histogram that indicates the distribution of DNA content among the cells in the tissue. In dividing cell population, two peak of DNA content are detected, one indicating cells in the G1 phase of the cell cycle and another for late S and G2 phases, after DNA replication. For image analysis microscopy, non-fluorescent dyes are used and the amount of dye bound to the individual cell is measured using a special software and microscope.

Analysis of DNA content has been associated with a certain amount of controversy. For example, normal liver may contain polyploidy (the state or condition of having more than two complete sets of chromosomes) populations and aneuploid (one or a few chromosomes above or below the normal chromosome number), such populations may be also found in both benign neoplastic and reactive soft tissue lesions. These findings complicated the interpretation of aneuploidy, and this clearly indicates that deviation from the usual diploid complement of chromosome does not equate to malignancy. Furthermore, non-cycling tumour placed in the culture may have S-phase DNA content. In practice, nuclei isolated from paraffin blocks are usually used to analyze the DNA content, and the results can be influenced by the fixative used and conditions of fixation. These situations have influenced the results of the studies on the DNA content which have yielded varying results.

2.10.2. DNA Ploidy

Ploidy is a cytogenetic term used to describe the number of chromosome sets or deviation from the normal number of chromosomes in a cell. In cytometry, the expression 'DNA ploidy' is used either to describe the DNA content in a cell or the total DNA distribution in a cell population (http://www.cancerbiomed.net/groups/hd/gallery/). An abnormal number of chromosomes in a single cell are known as Aneusomy. Loss of a single chromosome is called monosomy while gain of a single chromosome is called trisomy. In contrast, aneuploidy is defined as a condition of a population of cells when the average DNA content per cell in the cells comprising the G0/G1 phase is largely different from the normal diploid content (Ross et al., 2003).

A DNA index of 1.0 corresponds to a normal diploid (2N or 46 chromosome number) at G0 and G1 cells. The G2 and M cells feature a DNA index of 2.0 that corresponds to a 4N or chromosome number of 92 (Ross et al., 2003). In normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population forms the G0/G1 peak and 15% of the cells are in the S, G2 and M phases (Ross et al., 2003).

Normal non-cycling somatic tissue cells contain a constant amount (DNA-diploid) of nuclear DNA. By contrast, chromosomal abnormalities are virtually always present in malignant neoplastic cells which may also contain abnormal amounts of nuclear DNA, referred to as DNA aneuploidy. In the case of DNA-diploid tumours, a tumour cell population cannot be distinguished from normal cells on the basis of the DNA content (Spyratos, 1993).

DNA aneuploidy, also known as nondiploidy, is defined as a DNA content of the G0/G1 peak of a cell population that varies substantially from the G0/G1 peak of the known diploid reference cell population. The DNA index of an aneuploid cell population rarely might be less than 1.0 (hypodiploid) and far more commonly is greater than 1.0 (hyperdiploid). Nondiploid cell populations featuring a DNA index of the G0/G1 main peak at or near 2.0 must be differentiated from diploid tumours with significantly increased G2M phases. Some refer to these nondiploid cells with DNA indices near 2.0 as tetraploid tumours. This is because the nuclear DNA content of G1 nucleus reflects the ploidy of a cell. Hence, estimation of DNA content is frequently used for ploidy determination.

Table 2.1 Relation between the ploidy and DNA content of G1 phase nuclei

Ploidy	DNA Content (G1phase)
n	1C
2n*	2C**

* Diploid in terms of genetic content, ** Diploid in terms of the number of chromosomes

Euploidy is the state of a cell or organism having a basic multiple of the monoploid number, possibly excluding the sex-determining chromosomes. For example, a human cell has 46 chromosomes, which is a basic multiple of the monoploid number 23. Aneuploidy is the state of not having any euploidy. In humans, examples include; patients having a single extra chromosome (such as Down syndrome), or missing a chromosome (such as Turner syndrome).

The haploid number (n) is the number of chromosomes in a gamete of an individual and this is distinct from the monoploid number, which is the number of unique chromosomes in a single complete set. Gametes (sperm and ova) are haploid cells. The haploid gametes produced by (most) diploid organisms are monoploid, and these can

combine to form a diploid zygote. For example, most animals are diploid and produce monoploid gametes.

During meiosis, germ cell precursors have their number of chromosomes halved by randomly "choosing" one homologue, resulting in haploid gametes. Because homologous chromosomes usually differ genetically, gametes usually differ genetically from one another. Diploid (indicated by 2n=2c) cells have two homologous copies of each chromosome, usually one from the mother and one from the father, although all individuals have some small fraction of cells that display polyploidy. Human diploid cells have 46 chromosomes and human haploid gametes (egg and sperm) have 23 chromosomes.

2.10.2.1. Classification of DNA Ploidy

DNA ploidy has been classified into four types (Koss and Melamed, 2006):

1. Diploid: Cells have two homologous copies of each chromosome, usually one from the each parent. Nearly all mammals are diploid organisms. Human diploid cells have 46 chromosomes. The DNA index of diploid is equal to 1 (the ratio between DNA ploidy of the investigated tissue to the DNA ploidy of the well known diploid DNA). The histogram consist of single peak in 2C area and could be associated with a very small peak either of <10% of population within 5C (Raybaud et al., 2000) with the DI = 0.8-1.2.
2. Diploid-tetraploid: Various other deviations from normal diploid condition may occur that are neither triploid nor tetraploid. The DNA index of the tetraploid is 2, and that of triploid is 1.5. this group has varity of definition, in this study, this group had a diploid peak on histogram and not more than 25% of the population

in 4C (G2M area) in which will combine it with the diploid in measuring the ploidy of the margin of the tumour (Lanigan et al., 1993) with the DI = 1.8-2.2.

3. Aneuploid: Aneuploid may have DNA content either below normal (hypodiploid-aneuploid) or above normal (hyperdiploid-aneuploid). In this group notify several peaks of ploidy in area outside the diploid range even with small peaks in specific area on histogram could be considered as aneuploid (≥10% of population in 5C area) (Raybaud et al., 2000) with the DI = <0.8, >1.2<1.8, >2.2.
4. Aneuploid-tetraploid: within this group, the histogram presented in 4C with the DI of 1.8-2.2 and more than 25% of the population in 4C with the corresponding peak in 8C (Lanigan et al., 1993)

2.11. DNA Ploidy and Cancer Prognosis

DNA aneuploidy is often regarded as an indicator of tumour malignancy. Many early reports have indicated a high frequency of DNA aneuploidy in several human neoplasms. Tumours with hypertetraploid DNA content (DNA index > 2.20) were related to a worse, and those with a tetraploid DNA content to a better prognosis, than other DNA-aneuploid tumours (Kallioniemil et al., 1987). This has led to the detailed investigations of the influence of DNA aneuploidy on cancer invasion and metastatic progression and its potential for predicting prognosis.

Aneuploid primary tumours also recur more frequently as compared to diploid tumours (Giovagnoli et al., 1995). Sun et al. (1993a) have found that DNA ploidy can be used as an independent prognostic indicator for patients with stage A-C colonic adenocarcinomas. The survival of patients with prostatic adenocarcinoma has also been correlated with DNA ploidy (Azua et al., 1997). Similarly, Yuan et al. (1992) had

reported that DNA ploidy was significantly predictive of relapse of disease as well as overall survival in a series of patients with node negative breast cancer.

Azua et al. (1997) measured the DNA ploidy in fine-needle aspirates (FNAs) of breast cancer patients. They found that DNA quantification have significant relation to survival times. In another study involving a large series of breast cancer, Gilchrist et al. (1993) also found that DNA ploidy has strong correlation with lymph node metastasis and early death. DNA ploidy has also been correlated with the presence of tumour in the axillary lymph nodes, with distal metastases in a small series of breast cancer patients (Sherbet and Lakshmi, 1997). In a study done on gastric cancers, Baba et al. (2002) noticed that DNA aneuploidy was correlated with high invasive and metastatic potential. In another study on transitional cell bladder carcinomas by Loakim-Liossi et al. (2000), found a highly significant correlation between DNA aneuploidy and tumour recurrence. Similarly, aneuploid pancreatic carcinomas were found more aggressive than carcinomas with diploid DNA content.

Additionally, patients with diploid tumours have shown decreased median survival (Linder et al., 1995; Southern et al., 1996). Similar findings have also been reported in respect to colorectal cancer (Sampedro et al., 1996) and hepatocellular carcinoma (Bottger et al., 1996). Esteva et al. (2001), however, found no relationship between DNA ploidy and prognosis in lung cancer.

Similarly, neither overall survival nor disease-free survival of patients with head and neck cancers have no correlation with DNA ploidy (Tamas et al., 2000). Also noticed that there were no visible changes in the state of DNA ploidy during the progression of gastric cancers from early stages to the advanced stages of the disease. Thus, overall

there is little consensus concerning the value of DNA ploidy as a prognostic marker (Camplejohn et al., 1995) for poor prognosis.

Authors investigated DNA content and measure the ploidy of the needle aspiration fluid (Azua et al., 1997), fresh frozen or formalin fixed paraffin embedded tissues. Carrillo et al (1997) examined DNA content status by measuring the ploidy of 61 gastrointestinal tumours from needle aspirating fluids, fresh frozen and formalin fixed paraffin embedded tissues. Out of the 61 cases, they found 47 diploid cases, 12 aneuploid cases and 2 cases excluded due to uninterpretable histograms of the gastrointestinal tumours.

2.12. Distribution of DNA Ploidy in Oral Cancer

Baretton et al (1995) found from 106 cases of OSCC, 55% were aneuploid, 32% were diploid and 13% were tetraploid lesions. A high percentage of aneuploidy of OSCC was also observed by El-Rayes et al (2003) where their study showed 71% of 58 cases of OSCC were aneuploid with 29% being diploid. Similarly, Diwakar et al (2005) showed a high prevalence of aneuploid lesions where twenty-two (52.4%) out of 42 oral carcinoma had DNA aneuploidy, 1 with tetraploidy and 3 with diploidy, 16 monolayers with the eliminating 3 carcinomas were excluded because insufficient samples remined.

However, Garib et al (2002) reported in Iraqi patients a slightly lower prevalence of 41.7% aneuploid OSCC lesions as compared to 55% diploid lesions, with 2.7% being tetraploid lesions. In contrast, Melchiorri et al. (1996) investigated 25 OSCC where they reported only 5 were aneuploid (20%) with 17 peridiploid (hypodiploid) and three were hyperdiploid.

Measurement of the nuclear DNA content (ploidy) in patients with potentially malignant and malignant oral lesions as compared to normal controls showed 46% of the patients with potentially malignant lesions had diploid lesions, 37% had tetraploid

lesions and 17% had aneuploid lesions. DNA diploid tended to occur earlier in the progression from premalignant to malignant lesions and this may help in early detection of SCC where from lesions in high-risk groups, an examination of its ploidy status, and there was a significant difference between the patients with diploid and those with aneuploid lesions (Abou-Elhamd and Habib. 2006).

Vargas et al (2007) were found from 62 patients with pleomorphic adenomas that all had diploid DNA and only 17.4% were aneuploid, they noted that there was no correlation between gender, age, tumour site or size with ploidy status, with the two cases have cervical lymph node metastasis, one was aneuploid and the other case was diploid.

2.13. Ploidy and Prognosis of Head and Neck cancers

Abnormal DNA content has been associated with advanced stage of OSCC and other markers of poor prognosis, such as lower degree of differentiation and lymph node metastasis. It appears to be an independent prognostic factor for relapse and death; it was found useful also as a valuable differential diagnosis marker for nondysplastic oral white patches or as predictor of unclear nodal metastasis (Byers et al., 1998). In addition, Struikmans et al. (1998) have suggested that DNA aneuploidy and DNA tetraploidy could be used as a prognostic marker for head and neck squamous cell carcinoma.

Earlier researches on OSCC have found that there are only partial or no relation of the lymph node metastasis or tumour size with the ploidy level of the OSCC. Baretton et al, 1995 have found that ploidy status of the tumour cells did not show any correlation with patients' age, sex and grading (WHO). However, the pT stage have shown statistical significant with known follow-up in 90 patients, which suggested that it could be used

an indicator for prognosis. Melchiorri et al. (1996) also found that none of the clinical parameters (age, tumour site and size and lymph node status) was significantly associated with the DNA ploidy. Further, Melchiorri et al. (1996) suggested that the DNA content in primary tumours of the oral cavity and corresponding lymph node metastases may indicate that aneuploidy seems to take place before dissemination of metastatic cells. Baretton et al. 1995 showed that aneuploid tumours turned out to have lymph node metastases more frequently than diploid OSCC. In another study, Staibano et al. (1998) showed that the DNA aneuploidy in tumours is closely associated with their metastatic potential. By examining the 59 cases of malignant melanoma, Korabiowska et al. (2004) found that there were 6 cases euploid DNA, 32 cases were conspicuous for aneuploidy and the other 21 cases were clearly aneuploid.

Several other studies have shown that patients with diploid tumours were having decreased median survival (Linder et al., 1995; Southern et al., 1996). Previous studies have suggested that hyperdiploidy (slightly deviated after diploid histogram peak) could be associated with good prognosis and haploid state with poor outlook in childhood leukaemia (Wong et al., 2000). Presumably, it is not just the relationship between the general state of chromosome number or DNA ploidy to disease progression that one needs to take into account, but more importantly whether individual genes or cohorts of genes are amplified into multiple copies. It is not reasonable to assume that the chromosomal complement is amplified as a whole. But it is possible that specific chromosomes might be increased in number with each abnormal cell proliferation cycle. Abnormal cell cycle progression leads to hyperdiploidy.

CHAPTER 3: MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [**Ethical Approval Number: DF OS0902/0007(P)].**

The present study mainly consisted of four parts. The first part was to determine the prevalence of the ploidy status in OSCC by using DNA ploidy staining kit with Image Cytometry. The second part of the study compared the state of DNA ploidy of the tumour and the tumour margin. The third part had investigated the association of DNA ploidy of tumour margin and the pathological type of it. The fourth part had investigated the association of tumour DNA ploidy status with selected clinico-pathologic parameters.

3.1. Sample size estimation

Sample size estimation was done using PS sample size software for all study objectives. The fourth objective which was to determine the association between the state of DNA ploidy of the tumour and the clinicopathologic parameters has yielded the largest affordable sample size for this study considering the constraint in getting the cases and specimens. The software was used to calculate the parameters as below:

1. α = level of significant = 0.05.
2. $\beta = 0.25$
3. Power of study = $1 - \beta = 0.75$ (acceptable level of power of study).
4. Difference in state of aneuploidy which is significant to be detected = 0.30 = 30%, based on clinical impression.
5. m = ratio between negative (-ve) to positive (+ve) to the lymph node status.

The parameters were put into software and the sample size astimated was 80.

3.2. Sample selection

Oral squamous cell carcinoma (OSCC) of the cheek, upper and lower lip, alveolar mucosa, tongue and floor of the mouth were chosen as samples in this study. A total of 78 cases of histopathologically diagnosed OSCC between January 2002 to December 2009 (54 samples of surgical excision and 24 samples of incisional biopsy) were selected from the archives of the Oral Pathology Diagnostic Laboratory, Faculty of Dentistry, University of Malaya. Cases without margin (incisional biopsy) were included in this study together with the cases with margin (surgical excision) for the first and fourth objectives.

Following are the criteria considered for Inclusion of the samples for detecting DNA ploidy in OSCC:

a) Inclusion criteria:
    1. Specimens with ≥ 60% of tumour cells and /or epithelial cells of surgical margin (Buhmeida et al., 2006) – for all the objectives.
    2. Surgical margin, which are clear of tumour tissue ≥ 2mm– for 2nd objective.
    3. Complete socio-demographic, clinical and pathological data – for forth objective
b) Exclusion criteria:
    1. Specimens with < 60% tumour cells and/or epithelial cells of surgical margins – for all objectives.
    2. Surgical margin specimen, which are not clear of tumour tissue (involved margin) or <2 mm clear of tumour tissue. – For 2nd objective.
    3. Incomplete socio-demographic, clinical and pathological data - for 4th objective.

3.3. Study variables

3.3.1. Dependent variables

For all the objectives, DNA ploidy status was considered as dependent variable which was categorized into:

i. Diploid
ii. Diploid-Tetraploid (normal G2M)
iii. Aneuploid
iv. Aneuploid-Tetraploid

3.3.2. Independent variables

The variables considered as independent were:

a) Sociodemographic factors
   i. Age (≤50 years and >50)
   ii. Gender (Male and Female)
   iii. Ethnic groups (Malays, Chinese and Indians)
   iv. Habits (no & current/former)- smoking; betel quid chewing; alcohol drinking
b) Clinicopathological factors
   i. Tumour sub-site (lip, tongue, floor of the mouth, cheek, gum)
   ii. TNM staging (Stage 1 & 2; Stages 3 & 4)
   iii. Pathological grading (Well differentiated, moderately and poorly differentiated)
   iv. Pattern of invasion (Cohesive [Pattern 1 & 2] & Non-cohesive [Pattern 3 & 4])
   v. Tumour size (<2 cm, 2-4 cm, >4 cm)
   vi. Tumour depth (≤5mm and >5mm)
   vii. Nodal status (positive & negative)

3.4. Measurement tool

The present research was designed as a cross-sectional study which used formalin-fixed paraffin-embedded (FFPE) tissue samples of oral squamous cell carcinoma. The ploidy status of the specimens was done using Image-Pro MDA image cytometry (ICM) software and a series of calibration and validation was done to develop the ploidy criteria for the software. The overall steps involved in the calibration and validation process to ascertain the criteria for ploidy analysis using the Image-Pro MDA ICM is presented below in both explanation and as a flow chart (Fig 3.1):

1. Calibration of Flow Cytometry with DNA QC kit (standard for ploidy analysis).
2. Detection of ploidy status of peripheral blood monocyte (PBMC) using flow cytometry.
3. Calibration of the Image-Pro MDA ICM software with PBMC from the diploid DNA.
4. Validation of Image-Pro MDA ICM software against flow cytometry using selected formalin fixed paraffin embedded (FFPE) samples.
5. Development of the criteria for DNA ploidy using Image-Pro MDA ICM software.
6. Validation of newly developed criteria from Image-Pro MDA ICM software for DNA ploidy status with a commercial available ICM software OTMIAS (version 1.8, Olive Tree Media, Houston, USA).

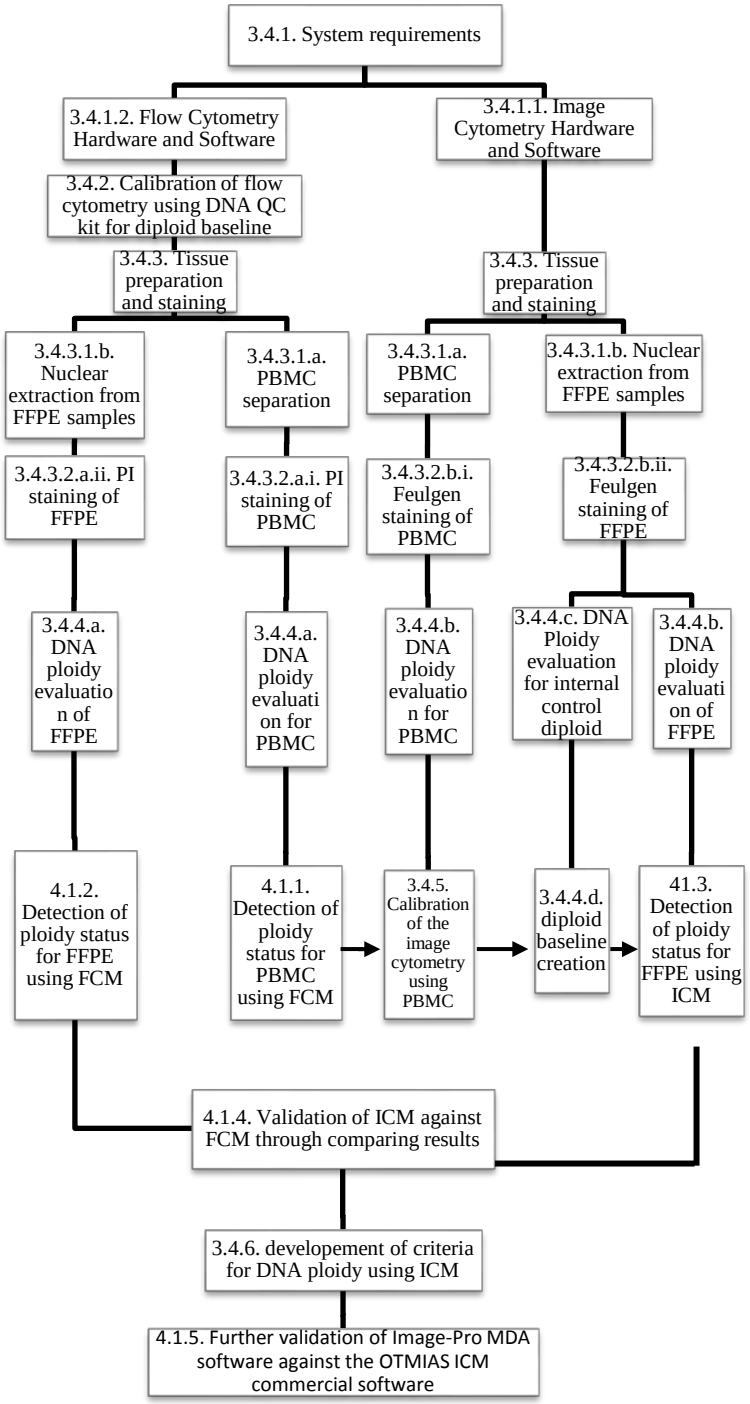


Figure 3.1. Flow chart for the development of the criteria for ploidy analysis using Image-Pro ICM.

3.4.1. System requirements

3.4.1.1. Image-Pro MDA Image Cytometry Hardware and Softwares

The Image Cytometric System consists of OLYMPUS BX61 motorized microscope and a computer Pentium 4 (window XP professional with special graphic card) and an LCD-monitor (Figure 3.2). For details of the characteristic properties of the Image-Pro MDA Image Cytometry System, see **Appendix1.**

Figure 3.2 Image Cytometry System

The image software used for the study was Image-Pro MDA. version 6.1.0.346 for window with serial 41N62000-53695 (Microlambda, SDN.BHD). A commercial software named OTMIAS, which was designed especially for DNA ploidy analysis was used to further validate the Image-Pro MDA ICM software after the validation with flow cytometry.

3.4.1.2. Flow Cytometry Hardware and Software

The BD FACSCalibur™ platform offers a unique modular approach to flow cytometry and allows users to perform both cell analysis and cell sorting in an innovative single bench-top system. The BD FACSCalibur dual-laser design provides the flexibility and sensitivity needed for multicolor analysis. Two lasers, an air-cooled argon laser and a

red diode laser, are spatially separated for high sensitivity. The BD FACSCalibur system works together with BD DNA analysis quality control products to eliminate many of the variables associated with ploidy and cell-cycle analysis. The instrument's optical and fluidic design ensures that high-resolution DNA ploidy and S-phase determinations are easy to achieve.

Flow cytometry equipment (FACSCalibur^{TM} from Becton Dickinson. USA. CA) used for the present study was from the Institute of Medical Research (IMR), Kuala Lumpur, Malaysia. The software used for ploidy analysis with flow cytometry was the BD CellQuest Pro software for general data acquisition and analysis.

3.4.2. Calibration of flow cytometry using DNA QC kit

The flow cytometry device was calibrated with a special DNA QC Kit (BECTON DECKINSON). For technical procedure refer to **Appendix 2** for calibration of the Flow Cytometry System.

3.4.3. Tissue preparation and staining

3.4.3.1. Tissue preparation

a. PBMC separation

Defibrinated or anticoagulant-treated blood was obtained from a volunteer and layered on the Ficoll-Paque PLUS solution. Density gradient centrifugation was applied for a short period of time to settle the monocytes in the bottom. The method for separation of PBMC from the blood in details is described in **Appendix 3**. The PBMC sample thus obtained was divided into two halves, one half was used for ploidy analysis in Image Analysis Cytometry and the other was used in Flow Cytometry. The PBMC was required for both techniques (image and flow cytometry) to create the diploid baseline for both.

b. Nuclear extraction from FFPE samples

Tissues sections (4 µm) were done and placed on each ordinary single end frosted slides for staining with H&E to confirm the presence of OSCC/epithelium in their respective blocks (for H&E staining refer to **Appendix 4**). This was done to determine and select the area of the tumour or the epithelium of the surgical margin for ploidy analysis. Three 50µm sections were dewaxed, rehydrated and the nuclei extraction was done using enzyme digestion. The technical details for nuclear extraction are described in **Appendix 5.** After all the 50µm sections were taken for ploidy analysis, one section of 4µm was cut and stained with H&E as the last reference slide. This was done, to ensure the presence of tumour cells and to verify whether the epithelial margin was placed in the glass test tube properly. Among the FFPE samples, 7 of the samples were used as calibration samples. Thus, after nuclear extractions, each sample nuclei were divided into 2, one to be used for calibration and the other for validation.

3.4.3.2. Staining

a. Propedium Iodide for FCM staining

i. PBMC samples

Normal blood lymphocytes were used as a diploid standard (Melchiorri et al., 1996). Control cells with known DNA content (PBMCs) were used to determine the DNA index (DI) of the test sample. For this the control cells were prepared using the same reagents as in the test sample. The DI was calculated from the ratio of the mode (or mean) of the G_0G_1 peaks of the test and reference (PBMC) populations for a particular specimen, following the protocol of Melchiorri et al. (1996). The detail procedure for the control sample staining with PI is mentioned in **Appendix 6**.

The PBMC cells that had been stained with the propidium iodide PI stain (Cycle TEST^{TM} PLUS DNA Reagent Kit, BIOMARKETING SERVICES (M) SDN.BHD, Kuala Lumpur, Malaysia) were used for the calibration and creation of diploid baseline.

ii. FFPE samples

The FFPE samples used for flow-cytometry analysis was obtained after following steps like dewaxation, rehydration and enzyme digestion, which were finally, filtered through mesh 50µm. The samples thus prepared were then placed in a special tube 17 x 100 mm for FCM analysis. As a control specimen mixed with PBMCs alone were considered, where at least a 2:1 ratio of tumour cells to PBMCs was used. The cells suspensions were centrifuged at 400g for 5minutes at room temperature (Cycle TEST ^{TM} plus DNA Reagent Kit, Cat No. 340242), then it was carefully decanted to remove the supernatant. The nuclei that remained in the bottom were stained with propidium iodide (PI) fluorescence stain. The detailed procedure is in **Appendix 7**.

b. Blue feulgen for ICM staining

i. PBMC samples

The other half of lymphocyte obtained from PBMC separation were stained directly with feulgen stain without enzyme digestion because the cytoplasm appear clearly during feulgen stain (translucent/no color). After washing the mononuclear cells (PBMC) once with medium, the cells were centrifuged at 400g for 5minutes. Then the supernatant was carefully decanted. To this, 2ml phosphate buffer saline (PBS) was added and then vortexed at medium speed. Again the samples were kept for cytocentrifugation (cytospine) at 600rpm for 8 minute followed by fixation at 10% buffered formalin saline for 60 min. Finally it was stained with blue feulgen. One of the PBMC slide after fixation in 10% buffered formalin and before starting the blue feulgen

stain was kept without acid hydrolysis (unhydrolysed slide), and then stained with blue feulgen stain. This was used as a negative control of the feulgen (Bocking et al., 1995). The technique details are as in **Appendix 8**.

ii. FFPE samples

The formalin fixed sections were dewaxed and rehydrated and then washed for three times in distilled water. Then the sections were, air dried and stored in a dust free environment at room temperature (18-25º C), until ready for staining with blue feulgen stain. Both the prepared PBMC and nuclei from FFPE samples were stained with ScyTek's Blue Feulgen Stain from (ScyTek Laboratories, Logan, Utah, U.S.A.) following the methods of Salim et al. (2008). Blue feulgen stain was designed to identify deoxyribonucleic acid (DNA) in cell nuclei. After staining the cells and nuclei were quantitatively evaluated for DNA content visually or using imaging cytometry systems.

3.4.4. DNA Ploidy evaluation procedures:

(a) PBMC cells and FFPE nuclei for FCM

The acquisition of cells or nuclei population for analysis using FCM was not less than 10,000 cells or nuclei for accurate results.

(b) PBMC cells and FFPE nuclei for ICM

The acquisition of PBMC cells and FFPE nuclei using both cytometry image software (OTMIAS and Image Pro MDA) were done on a minimum of at least 250 cells or nuclei (**Appendix 9**).

(c) Lymphocytes for internal control (normal diploid)

Lymphocytes in the same tissue sections (FFPE samples) were used as internal control (normal diploid standards) in the cytospin preparations of FFPE tissues (Dorman et al.,

1990). OTMIAS software was calibrated on Diploid baseline. The detailed procedure for analysis using the OTMIAS software is described in **Appendix 10**.

(d) Analysis using Image-Pro MDA

The Image-Pro MDA is multipurpose software and it can be used to measure the Integrated Optical Density (IOD), which is a very important variable in measuring the DNA content, and in determining the ploidy status of the samples. Since this is the first attempt to use the IOD from the Image-Pro MDA software to measure DNA ploidy, there was a necessity to calibrate and create a Diploid baseline for the Image-Pro MDA software. The readings obtained through Image-pro MDA with PBMCs images were used to determine the diploid baseline for further sample analysis.

3.4.5. Calibration of the Image-pro MDA image cytometry using PBMC

Before started using the Image-pro MDA image cytometry system for capturing the images of cells or nuclei and then for doing analysis, the system was first prepared as described in **Appendix 11** (Bocking et al., 1995). The calibration of the diploid baseline for the Image-Pro MDA software was based on the comparison of the PBMC analysis using the results from Image-Pro MDA and that of the same results from PBMC analysis using FCM and OTMIAS software. The detailed procedures using the Image-pro MDA software for DNA ploidy analysis is described in **Appendix 12**.

3.4.6. Development of criteria for DNA ploidy using Image-Pro MDA image cytometry

Based on the calibration of the FCM and ICM (Image-pro MDA) using external/internal control and validation of the ICM against the flow cytometry, the final criteria for determining the DNA ploidy status using the Image-Pro MDA ICM was created as a set of guidelines, which is mentioned below:

According to the DNA IOD and DI:

- Diploid DNA content 2C = 750-1350 IOD which has DI = 0.8-1.2.

- Diploid-Tetraploid DNA content 4C=1500-3000 IOD, less than 25% of cells in 4C and has DI = 1.8-2.2. (represent the G2M cell cycle)

- Aneuploid-tetraploid DNA content 4C = 1500-3000 IOD, more than 25% of cells in 4C with the corresponding peak in 8C and has DI = 1.8-2.2.

- Aneuploid DNA content = (a) DNA >1351 IOD (b) DNA < 749 IOD which has a
  DI= <0.8, >1.2<1.8, >2.2.

3.5. Data Acquisition/collection:

The following data were obtained from the Malaysian Oral Cancer Database and tumour bank system (MOCDTBS) coordinated by Oral Cancer Research and Coordinating Centre (OCRCC) (**Appendix 13**).

- Sociodemographic data - age, gender, ethnicity, and habits.
- Clinicopathologic data - TNM staging (AJCC, 5th Edition), histological grading, pattern of invasion, tumour size, nodal status, depth of invasion and distant metastasis.

3.5.1. Histopathological and clinicopathological criteria

a. Broders' tumour grading

This grading system subjectively assesses the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity of the tumour cell population (Pindborg et al., 1997). Tumour was graded as grade 1, 2 and 3 as in **Appendix 14**

The margin of the tumour was classified into clear margin (5 mm and more from tumour) and close (1-4 mm from the tumour) (Woolgar and Triantafyllou. 2005; Helliwell and Woolgar, 1998).

3.6. Evaluation of cells/nuclei for inclusion in the analysis for ICM

i. Criteria for DNA staining and grading of stained cells

A homogeneous staining or a speckled/dotted pattern in the nucleus is considered as a positive/true staining and the absence of distinct nuclear staining is taken as negative/false for staining

ii. Criteria for counting of stained cells

Five areas on slides were selected for image capturing and at least 250 nuclei were selected for the tumour and margin analysis and as internal control, 40–50 lymphocytes from the same sample slides were selected.

3.7. Statistical analysis

Categorical data was statistically analyzed by Chi-square and Fisher's exact test. The continuous data were summarized as mean ± SD (standard deviation). Significance was assessed by Chi-square and McNemar test. Level of significance were kept as $P < 0.05$.

CHAPTER 4: RESULTS

The sociodemographic characteristics of the 78 samples of OSCC with a mean average age of 59±12.26 years were summarized in Table 4.1. Majority of the sample was female (69.2%; n=54), with the predominant ethnic group being the Indians (71.8%; n=56). The data also revealed that majority of the subjects indulged in habits like betel quid chewing, smoking and alcohol drinking habits. The most affected site was the cheek (51.3%; n=53) and the tongue (29.5%; n=23).

Table 4.1 Socio-demographic Characteristics of Samples

Variables n = 78		**No of samples (%)***
Age group Age Mean±SD (59.73±12.264) Age Range: 26-94	<=50	19 (24.4)
	>50	59 (75.6)
Gender	Female	54 (69.2)
	male	24 (30.8)
Ethnicity	Malay	12 (15.4)
	Chinese	10 (12.8)
	Indian	56 (71.8)
Smoking	Never	43 (55.1)
	Former, stop	35 (44.9)
Alcohol	Never	59 (75.6)
	Former, stop	19 (24.4)
Betel quid chewing	Never	18 (23.1)
	Former, stop	60 (76.9)
Site	Cheek/gum	53 (67.9)
	Tongue/floor of mouth	23 (29.5)
	Lip**	2 (2.6)

*Chi-square test; **excluding the lip

4.1. Development of criteria for determining ploidy status using the Image-Pro MDA

4.1.1. Detection of ploidy status for PBMC analysis using FCM

Normal human blood lymphocytes (PBMC) were used as diploid standards. DNA ploidy values were expressed in C units (diploid DNA content = 2C) (Melchiorri et al,. 1996). The measurements were performed on FACS Caliber (Becton Dickinson, USA, CA), supplied with an argon laser with excitation wavelength of 488nm. The first peak on the left of the histogram was considered to represent diploid G0-G1 cells (PBMC showed a single peak of the Diploid of PBMCs -Figure 4.1). Histogram with coefficient of variation (CV) less than 8% were considered of good quality (Schutte et al., 1985).

To set the diploid peak as a control in the histogram of the tumour samples, PBMCs was mixed with the tumour samples.

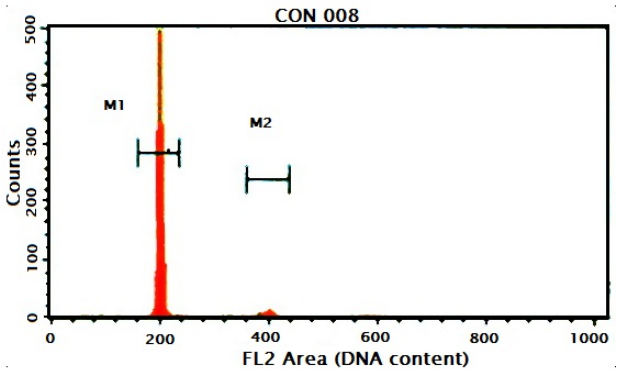
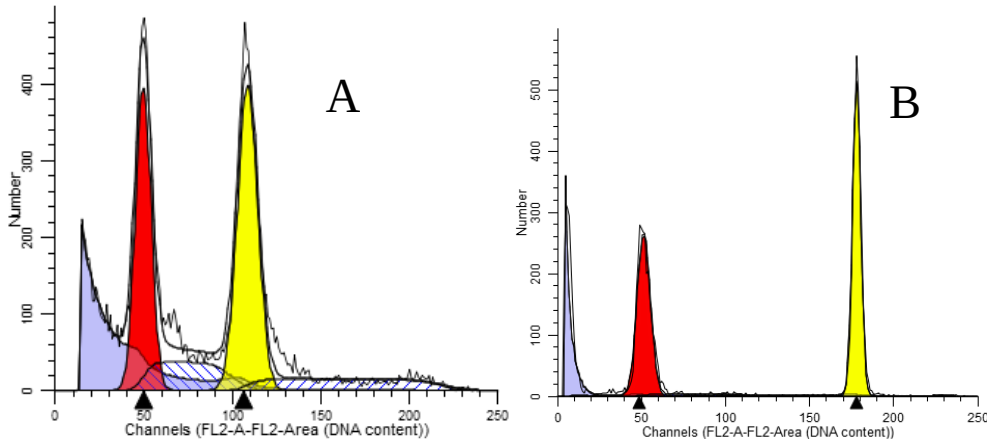


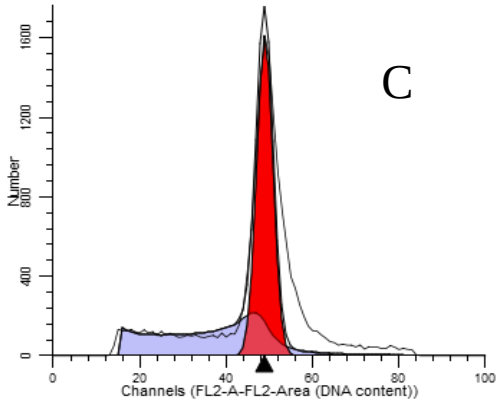
Figure 4.1 Histogram of diploid PBMC in 2C Diploid, the linearity of the PBMC linearity (M2/M1) = 1.98, resolution (CV of M1) = 2.09

4.1.2. Detection of ploidy status for FFPE analysis using FCM

Altogether 7 FFPE samples were analyzed by flow cytometry to determine the ploidy content. Analysis of FCM was done using the MODFIT software from Institute of Medical Research/Malaysia. Figure 4.2 showed the histogram for ploidy status analysis using FCM.



A-Tetraploid = DI > 2 B-Aneuploid= DI > 1.15



C-Diploid= 1 < DI< 1.15

Figure 4.2 Histogram of ploidy status of FFPE samples using FCM. A: Tetraploid DI >2.0, B: Aneuploid DI > 1.15, C: Diploid DNA in which 1<DI<1.15, the CV of the three pattern of DNA ploidy status was less than 8.

The criteria for DNA ploidy analysis by Flow Cytometry was considered as according to EL-Rayes et al. (2003):

1) Tetraploid DNA: Single DNA histogram revealed a DI>2.0.
2) Aneuploid DNA: Histogram with single or multiple G0/G1 peaks having DI > 1.15.
3) Diploid DNA: Single G0/G1 peak of DI = 1 < DI< 1.15.

The results were obtained by entering the data in MODFIT software and FLOWING software from flow cytometry analysis.

Table 4.2 showed the ploidy status with the accompanying CV of G0/G1 and DI of the 7 FFPE samples. The CV for all selected tumours were < 8 except in case No.5 which showed a higher limit due to heterogeneity of the tumour, and the DI in case No. 14 showed lower limit compared to the DI of tetraploid (> 2).

Table 4.2 Ploidy content of 7 FFPE samples and the coefficient of variants (CV) and DNA indices (DI) using MODFIT software for the flow cytometry.

Case No.	Ploidy level	CV of G0/G1 %	DI
5	Aneuploid	9.23	4.73
14	Tetraploid	5.05	1.90
20	Diploid	3.79	1.12
36	Aneuploid	1.37	3.45
58	Diploid	6.69	1.02
67	Tetraploid	5.31	2.18
76	Aneuploid	5.93	5.01

4.1.3. Detection of ploidy status of FFPE using ICM

The techniques using IOD and DI for Image-pro MDA feulgen-thionin-stained slides (blue feulgen) were used to quantify nuclear DNA content. After DNA staining, the IOD is considered as the cytometric equivalent of its DNA content. The DNA histograms were classified as diploid and aneuploid DNA based on the amount of DNA relative to the normal control reference (the diploid position (2C) determined after calibration of the system). The normal 2C reference values were established by measuring the normal well-preserved lymphocytes in each slide as internal reference cells, whose CV was < 12.5% (Salim et al., 2008).

The Image-Pro MDA software was calibrated with the external control (PBMC) lymphocytes by using the same lymphocytes images. This was actually the second half of PBMC used for analysis by FCM and is regarded as the External reference PBMC IOD for diploidy. The IOD of 250 blue feulgen stained nuclei of the PBMC was

obtained from the Image-Pro MDA software where the IOD of External reference PBMC IOD for diploidy was found to be between 753 - 1302. For the evaluation of FFPE case, 50 internal lymphocyte (used as the internal control) and 250 tumour cells were analyzed. Since tumour cell specimens contained benign cells admixed with cancer cells, the latter were selected based on cytologic criteria of malignancy (Raybaud et al., 2000). After calibration of the software with PBMC IOD (which lie in between 753 – 1302) and the measurement of IOD of internal lymphocyte, it was determined that the number of IOD for diploid would be within the range of 787-1323.

Therefore the following criteria can be applied for the 2C, 3C, 4C, and 5CER (5C exceeding rate), the histogram generated by the Image-Pro MDA:

1. The diploid 2C on histogram represent the IOD between 750-1350 according to IOD of the PBMC range
2. Tetraploid 4C on histogram represent the double of IOD of the diploid which is 1500-3000
3. The areas on histogram between 2C-4C, which represent the fraction phase of diploid and the aneuploid have IOD 1351-1499
4. The areas on histogram above the high limit of 4C will be considered as the aneuploid level of cell which have the IOD of more than >3001. 5C and 5CER (5C exceeding rate)
5. The events that have IOD less than the 2C have the IOD < 750, which was considered as hypodiploid/aneuploid.

The formula for determining the DNA ploidy by ICM was dependent on IOD as follows (Raybaud et al., 2000):

1. Aneuploid = (a) ≥10% in 5C or, (b) ≤10% in 5C but ≥50%in 2.5C
2. Diploid = (a) <10% in 5C and <50% in 2.5C

4.1.4. Validation of ICM against FCM

To validate the above-mentioned method of classification depended on DI (DNA index = mean IOD of tumour / mean IOD of internal control). The ploidy criteria using DNA Index had been categorized by Lanigan et al., (1993) as follows:

1. Diploid = DNA index between 0.8-1.2
2. Diploid-Tertraploid = DNA index more than 25% of cells at 4C with the DI in the range 1.8-2.2
3. Aneuploid-Tetraploid = DNA index more than 25% of cells at 4C with the DI in the range 1.8-2.2, with the corresponding peak in 8C
4. Aneuploid = DI < 0.8, DI > 1.2 < 1.8, and DI > 2.2.

Analysing the image cytometry images, which were captured from PBMCs stained with blue feulgen showed that more than 95% of the nuclei (250 nuclei) in 2C (G0/G1) phase with CV of 8.73% have a DI gain of 1.04 in Image-Pro MDA software.

The result of comparing the IOD of the PBMC (external control) for calibration of the ICM, and the cutoff point of the diploid DNA (2C), which is in between 750-1350, was effective and obtain an accurate result in comparing the DI of the Diploid DNA in published paper (DI= 0.8-1.2) (Lanigan et al., 1993). The most important step in the calibration and validation of the Image-Pro MDA software was distinguishing the normal diploid from the abnormal aneuploidy-tetraploid (Figure 4.3).

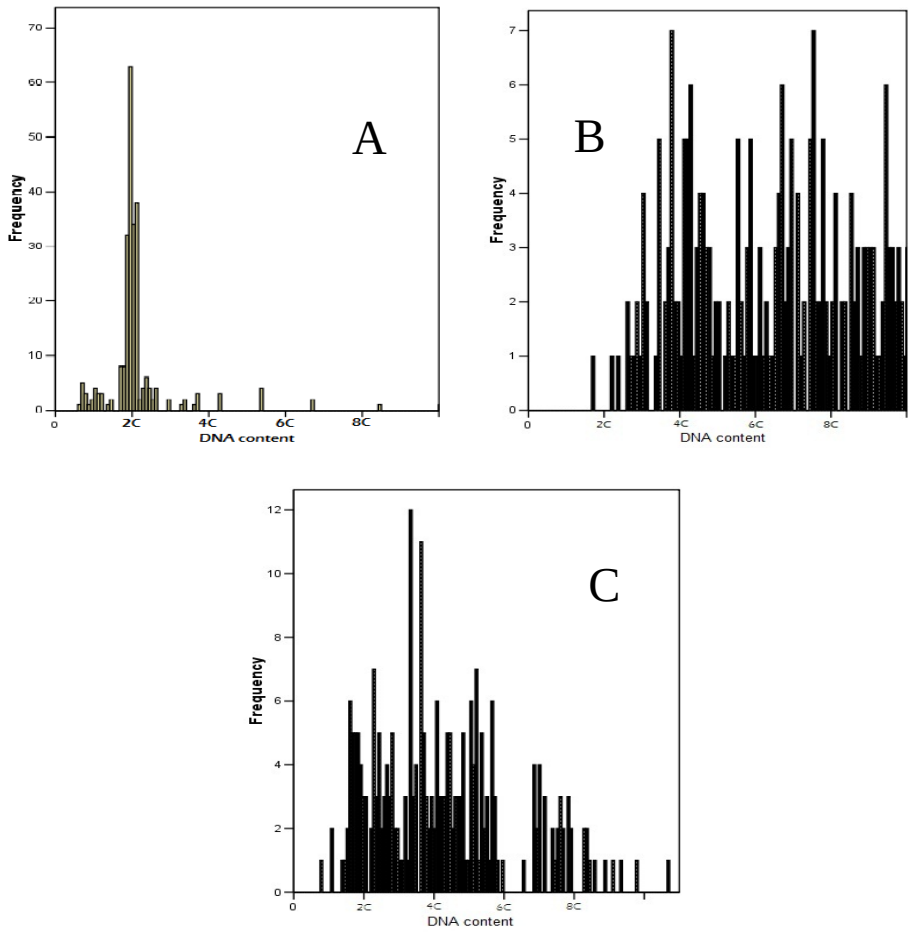


Figure 4.3 Histogram of ploidy status of FFPE samples using Image-pro ICM. A: Diploid tumour which had a peak on 2C and <10% in 5C and <50% in 2.5C with the DI of 1.17 and CV of G0/G1 2.62; B: Aneuploid tumour which had a peak on more than 10% in 5C, with the DI of 4.28 and CV of G0/G1 3.93; and C: Aneuploid-Tetraploid tumour which had more than 25% of population in area 4C with the corresponding peak on 8, with the DI of 2.01 and the CV of G0/G1 4.20.

Finally, the results from the Image-pro MDA ICM were compared with the FCM results using the 7 test samples. The comparison of ploidy status between FCM and Image-pro ICM is as in Table 4.3. In this table the DNA tetraploidy of the tumour status was included within the aneuploid because it represented the abnormal ploidy status as all of them were (tetraploid) not representing the cell cycle phase (not G2M). The DNA ploidy status of 7 FFPE using the Image-pro MDA image cytometry software showed concordant with the FCM. The only difference is that in FCM the case No. 14 and 67

showed tetraploidy while in ICM showed aneuploid. This difference was due to the higher sensitivity of ICM in differentiating the aneuploidy and tetraploidy.

Table 4.3 Comparison of the FCM and Image-pro MDA ICM results of 7 test samples.

Case No.	FCM	ICM / Image Pro.
5	Aneuploid	Aneuploid
14	Tetraploid/ Aneuploid	Aneuploid
20	Diploid	Diploid
36	Aneuploid	Aneuploid
58	Diploid	Diploid
67	Tetraploid/ Aneuploid	Aneuploid
76	Aneuploid	Aneuploid

The DNA ploidy status results for both ICM (Image-pro MDA) against the FCM showed 100% concordant if the tetraploid from the FCM results of case No.14 and No.67 was accepted as aneuploidy. The tetraploid found in FCM in these cases do not represent the G2M phase of the diploid fraction phase (Lanigan et al., 1993) because all these are tumours and which are differentiated from normal G2M. This was done based on the definition that ‘Tetraploidy = tumour having more than 25% of cells at 4C with the DI in the range 1.8-2.2, and with the corresponding peak at 8C. The tetraploid in our classification criteria represents the abnormal phase from normal diploid and thus according to this the tetraploid was considered to be included within the aneuploid.

4.1.5. Validation of DNA ploidy status from Image-Pro MDA software against the OTMIAS ICM commercial software

A further validation of the results from the Image-Pro MDA using the newly developed criteria for Image-pro MDA ICM software was further validated against the OTHMIAS ICM commercial software. The results from Image-Pro MDA and OTMIAS ICM

softwares are summarized in Table 4.4. Both software gave the same results for all 7 samples and thus recording a 100% concordance between the Image-Pro MDA software and OTMIAS.

Table 4.4 Ploidy status of 7 samples using Image-pro MDA and OTMIAS software.

Case No.	**Image pro**	**OTMIAS**
5	Aneuploid	Aneuploid
14	Aneuploid	Aneuploid
20	Diploid	Diploid
36	Aneuploid	Aneuploid
58	Diploid	Diploid
67	Aneuploid	Aneuploid
76	Aneuploid	Aneuploid

4.2. Prevalence of the state of DNA ploidy in OSCC

For the tetraploid and aneuploid tumour, DNA ploidy was considered together. They were grouped into aneuploidy tumour (abnormal DNA), because the tetraploid DNA do not represent the G2M of the cell cycle but it represented the abnormal DNA as it had more than 25% of the population in 8C area. Majority of the tumours were aneuploid (96.2%) and only 3.8% of tumours were diploid (Table 4.5). Among the aneuploid tumours, 51.3% were tetraploid tumours (Table 4.5).

Table 4.5 Prevalence of DNA ploidy status of OSCC showing distribution of the aneuploid-tetraploid and aneuploid tumour groups

Total no. of tumours n=78	Diploid tumours n (%)	Aneuploid tumours n (%)	
		Tetraploid n (%)	Aneuploid n (%)
All Tumours	3 (3.8)	40 (51.3)	35 (44.9)

4.3. Comparing DNA ploidy status between the tumour and its margin

Diploid and the tetraploid DNA was considered as normal cell cycle phase for margins. They were combined and were considered as the normal diploid. When the tetraploid were not showing peak of 25% at 8C, they were considered as normal diploid margin while the other part with aneuploidy represents the aneuploid margin.

Table 4.6 showed that the tumor DNA ploidy status was significantly associated with the ploidy status of the margins (McNemar test) where all the diploid tumours were associated with diploid margins while among the aneuploid tumours, 15.7% were aneuploid margins.

Table 4.6 Comparison of DNA ploidy status of tumour and its margin

n=54		Margin DNA ploidy status		*P value**
		Diploid margin** n (%)	Aneuploid margin*** n (%)	
Tumour DNA ploidy status	Tumour diploid n (%)	3(100)	0 (0)	<0.001
	Tumour aneuploid n (%)	43 (84.3)	8 (15.7)	

*McNemar test; **Diploid margin includes diploid and the diploid-tetraploid DNA (normal G2M of the normal cell cycle); ***Aneuploid margin includes aneuploidy and aneuploidy-tetraploid DNA.

Table 4.7 compared the pathological types of surgical margins with the tumour ploidy status. All the close margins were associated with aneuploid tumours while among the clear margins, a high percentage (92.1%) were associated with the aneuploid tumours ($p < 0.001$).

Table 4.7 Comparison of DNA ploidy status of tumour with pathological types of surgical margins

n=54		Tumour DNA ploidy status		Total n (%)	*P value**
		Diploid tumour n (%)	Aneuploid tumour n (%)		
Pathological types of surgical margins	Clear Margin	3 (7.9)	35 (92.1)	38 (100)	<0.001
	Close Margin	0 (0)	16 (100)	16 (100)	

* Chi Square test

4.4. Comparison of DNA ploidy status of surgical margins with the pathological type of surgical margins

Table 4.8 compared the DNA ploidy status of the surgical margins with the pathological tyopes of surgical margins. Most of the clear margins were diploid, however 15.8% of the clear margin was aneuploidy. Similarly, most of the close margins were diploid with 12.5% aneuploids. However, there were no statistical significant in the relationship between margin DNA ploidy status and the pathological types of surgical margins (Table 4.8).

Table 4.8 Comparison between the Margin ploidy status and the pathological types of surgical margins (close and clear margins)

n=54		Margin DNA Ploidy Status		Total	*Pvalue*
		Diploid margin n (%)	Aneuploid margin n (%)		
Pathological types of surgical margins	Clear Margins	32 (84.2)	6 (15.8)	38 (100)	0.75
	Close Margins	14 (87.5)	2 (12.5)	16 (100)	

*Chi Square test

4.5. Association between the state of DNA ploidy of the tumour and the sociodemographic and clinicopathological parameters

Table 4.9 compares the different sociodemographic and clinicopathologic variables with the Tumour ploidy status.

As illustrated in the table 4.9, 4 cm was taken as the cut off point to compare the tumour size. The selection of 4cm as a cut off point was considering T (tumour size) as the TNM staging where T1 (<2cm) + T2 (2-4cm) and T3 + T4 (>4cm) are different groups.

The selection of 5mm as a cut off point for depth of invasion was according to the classification done by Speight and Morgan, 1993 and Fukano et al. 1997.

Patients with age more than 50 years of old were found mostly with aneuploid tumour, it represented 73% of the whole patients (n=78). Among the female, 96.3% were found to have aneuploid tumours. Ethnicity showed that among the Indians, 96.4% have aneuploid tumours. From the habit analysis, the patients with betel quid chewing habits have maximum aneuploid tumour (96.7%). However, there were no statistical significance between tumour ploidy status and the above parameters (Table 4.9).

There was significant difference in the ploidy status of tumours and their pattern of invasion, tumour site, histopathological classification and depth of invasion (*p=0.004, p=.009, p=0.02, and p=0.004*) respectively (Table 4.9). Among the tumour size, all the patients who had tumour more than >4mm were reported as aneuploid tumour. All patients with moderately and poorly differentiated OSCC had aneuploid tumour. Among the tumours with non-cohesive pattern of invasion, 89.5 % were aneuploid tumours. Among the tumour site, all the cheek and gum tumours were aneuploid tumour and among the depth of invasion of tumours, all tumours with >5mm depth were aneuploid tumours (Table 4.9).

Table 4.9 Association of tumour DNA ploidy status with sociodemographic and clinicopathological parameters

Variables		Tumour DNA Ploidy Status		*P value**
		Diploid % (n) N=3	Aneuploid% (n) N=75	
Age group n = 78	<=50	1 (5.3)	18 (94.7)	0.712
	>50	2 (3.4)	57 (96.6)	
Gender n = 78	Female	2 (3.7)	52 (96.3)	0.922
	male	1 (4.2)	23 (95.8)	
Ethnicity n = 78	Malay	1 (8.3)	11 (91.7)	0.587
	Chinese	0 (0)	10 (100)	
	Indian	2 (3.6)	54 (96.4)	
Smoking n = 78	Never	1 (2.3)	42 (97.7)	0.439
	Currently, stop	2 (5.7)	33 (94.3)	
Alcohol n = 78	Never	1 (1.7)	58 (98.3)	0.082
	Currently, stop	2 (10.5)	17 (89.5)	
Betel quid chewing n = 78	Never	1 (5.6)	17 (94.4)	0.667
	Currently, stop	2 (3.3)	58 (96.7)	
Histopathological classification n = 78	Well	3 (10)	27 (90)	0.025
	Moderate & Poor	0 (0)	48 (100)	
Pattern of invasion n = 78	Cohesive 1,2	2 (20)	8 (80)	0.004
	Non-cohesive 3,4	1 (1.5)	67 (98.5)	
Tumour site** n = 76	Cheek/gum	0 (0)	52 (100)	0.009
	Tongue/floor of mouth	3 (12.5)	21 (87.5)	
Pathological TNM*** n = 54	Stage 1,2	2 (8.7)	21 (91.3)	0.386
	Stage 3,4	1 (3.2)	30 (96.8)	
Tumour size*** n = 54	<=4 cm	3 (10)	27 (90)	0.111
	>4 cm	0 (0)	24 (100)	
Lymph node metastasis*** n = 54	negative	2 (5.9)	32 (94.1)	0.891
	positive	1 (5)	19 (95)	
Depth of invasion*** n = 54	=<5mm	3 (20)	12 (80)	0.004
	>5mm	0 (0)	39 (100)	

*****Chi Square test **lip was excluded from analysis. ***only tumour with margin was included here.

CHAPTER 5: DISCUSSION

5.1. Sample size

The total of 78 samples selected for the present study were screened from the total of 121 OSCC cases that were diagnosed at the Oral Pathology Diagnostic laboratory Faculty of Dentistry, University of Malaya between January 2002 till December 2009. The small sample size in this study compared to the other published paper was mainly due to the strict criteria used for the sample selection, so that to avoid any biasness in the results during ploidy analysis. For example, one of the main criteria which was chosen that is the presence of more than 60% tumour tissue have limited the sample number to be selected from the available formalin fixed paraffin embedded 121 OSCC samples. Many other researchers who have done on ploidy analysis in OSCC tissues have also reported only a small sample size. A recent study by Brandizzi et al. (2009) have selected around 67 cases of primary OSCC, where as Staibano et al. (1998) recorded 25 cases, but it was on resected OSCC samples. Furthermore, Diwakar et al. (2005) have recorded only 45 cases of untreated primary OSCC, whereas Vargas et al. (2007) have recorded slightly more of 62 cases. Therefore the assumption is that the sample size taken (78 cases) in the present study will not interfere with the overall results in DNA ploidy analysis.

5.2. Development of DNA Ploidy Criteria using Image cytometry

Many studies have indicated that the analysis of DNA ploidy could be opted as a better method for the detection of early tumoural lesions or at high risk of tumour progression (Fang et al., 2004; James and Atkinson, 1989; Khan et al., 1994; Reid et al., 2000). Previous studies have shown that the DNA ploidy analysis was helpful in identifying the aggressiveness of malignant neoplasm in a variety of organs including; prostate, breast, urothelial tract, cervix, ovary, lung, skin and oral mucosa (Berchuck et al., 1992;

Yamamoto et al., 1994; Mainguene et al., 1997; Talve et al., 1997; Haroske et al., 2001; Kay et al., 2002; Kristensen et al., 2003).

Image cytometric DNA analysis (ICDA) is an optical technique allowing visualization of abnormal nuclei that may be undertaken on archival tissue. DNA ploidy abnormalities measured by image cytometry have been suggested as one of the strong predictors of future cancer development (Dunn et al., 2010). Interestingly the diagnosis of aneuploidy by image cytometry has not been standardized so far. One of the hindrances to the standardization is because of the difference in image cytometry systems used in laboratories, DNA staining techniques, section thicknesses, and types of control cells. Though many image cytometry systems have been used by many researchers are the CAS 100 (Mairinger and Gschwendtner, 1998; Gschwendtner et al., 1999; Reid et al., 2000; Keller et al., 2001; Dreinhofer et al., 2002; and CAS 200-Cell Analysis System Image Analyzers (Becton-Dickinson, San Jose, CA) (Reid et al., 2000; Fang et al., 2004;), the QPATH (LEICA, Cambridge, England) (Baak et al., 2003), the MPV3 (Leitz, Wetzlar, Germany) equipped with a DNA cytometry system (ACAS, Ahrens, Bargteheide, Germany) (Keller et al., 2001), the SAMBA 4000 Image Analyzer (Imaging Products International, Chantilly, VA) (Jiang et al., 2003), and the Firefield ploidy system (Firefield Imaging Ltd, Nottingham, UK) (Kristensen et al., 2003). Huang et al (2005) showed that the performance of these instruments has not been compared systematically and there is little published data comparing them.

Hence, the main focus of the ICM criteria development was to finally compare the results of IOD and DI analysis obtained from the ICM and FCM systems and thus to ascertain the accuracy of using ICM in detecting DNA ploidy level. We found that though the DI obtained from both the systems were well correlated, but the DI values

obtained with the ICM system was slightly higher than those acquired from the FCM. We also found that the ICM was more sensitive in diagnosing aneuploidy, which was in agreement with other researchers who compared both the systems (Baretton et al., 1995; Abou-Elhamd and Habib. 2006; Bremmer et al., 2008). Several other studies on solid tumors also proved that the image cytometry to be more sensitive for diagnosing DNA content abnormalities (aneuploidy) than flow cytometry (Huang et al., 2005). Many lesions diagnosed as euploid on flow cytometry have been found to be aneuploid on image cytometry (Pindur et al., 1994; Alanen et al., 1998). In OSCC lesions, however, both flow and image cytometry have been used in the diagnosis of large series of patients (Baretton et al., 1995). Image cytometry performed on histological sections allows selective examination of the targeted epithelial cells (Huang et al., 2005). Small aneuploid populations are detected by computed image cytometry which may be masked by the large numbers of benign or inflammatory cells included blindly in a flow histogram. It must be pointed out that the small number of cells analyzed in a computed image cytometry system magnifies the effect of sample error. Cells in a computed image cytometry preparation may be overlapping or damaged, and therefore will be rejected by the operator. This potential disadvantage may be overcome, to an extent, by software in the future updating of image analyzers which can electronically subtract overlapping images.

Though very accurate and efficient one of the drawback which we observed by using ICM was that the consumption of time for the whole procedure, which was far more than the FCM. However, the DI values obtained by the ICM were highly reproducible, which suggests the necessity of using such systems in cancer diagnosis. An interesting observation was observed on DI values obtained between the ICM and FCM, where we found that values were higher in ICM than those obtained using the FCM. There are

several possible explanations for this variation; difference in cytometric methods applied to assess nuclear DNA content (image or flow), type of tissues assessed (tongue, oral cavity, head and neck region), the method of tissue preparations (fresh, fixed), or to the various methods used to define ploidy. Garib et al. (2002) have suggested that there is difficult to point out one particular method that can provide the best result for DNA ploidy content from a variety of tumours or tumours from different sites.

Moreover, ICM results may get influenced depending upon nuclear morphological features; including nuclear area, shape and density. It could be argued that the selection of control cells could influence the result, but they may not make a dramatic difference in DI determination unless the controls are actively dividing cells such as gastrointestinal epithelial cells (Huang et al., 2005). It is therefore unlikely that the selection of control cells can explain the significant differences in the DI values generated by these two systems. However, to resolve all these parameters which could influence the result, the extra software validation system made utilized for DNA ploidy analysis in the present study were proven to be advantageous. The CV and the DI on the both ICM software were convergent, and the ploidy level in both were concordant.

One of the main difficulties faced by those who seek to make use of analytic cytometry is the lack of standardized rules for histogram interpretation. Hence the current study has attempted to reconcile the results provided by computed image cytometry and flow cytometry. The question of maximum permissible count in the G2M phase of a cycling diploid population is always problematic. Moreover, the differences in software applied for calculation probably accounted for the discrepancies in the figures quoted for this variable. In other studies also the researchers have suggested a variability range of 5-20% due to the calculation using software (Rainwater et al., 1987; Layfield et al., 1989;

Lanigan et al., 1993; Currin et al., 1990). Generally, difficulty arises in differentiating the ploidy class (diploid vs tetraploid) using flow cytometry. Further, cases with G2M phases more than 20% on flow cytometry were confirmed to be tetraploid by computed image cytometry in all the attempts during the present study except two instances. These two instances occurred during the study, we suspect that sample error might have accounted for the failure of computed image cytometry, which were judged by confirming the length of 4C peaks (quite large). In our study few nuclei which were showing DNA content of 8C was considered overlapped or damaged by the system and might have excluded from the histogram. This has notice to be one of the drawbacks of the system, where the possibility of even small exclusion of nuclei could influence the result. Difficulty in ploidy categorization by flow cytometry when G2M phase proportions are at the upper limit of diploid values will probably persist, regardless of the method applied to the calculation.

There are insufficient data on how hypodiploid aneuploidy is categorized in FCM or ICM. Whether it represents the biological characteristic which are distinct from hyperdiploidy is not known. Certainly there is evidence from cytogenetic studies that hypodiploidy is relatively a common feature for many solid tumours and that small hypodiploid populations may be missed during flow cytometry analysis (Shackney et al., 1990). However, image analysis along with other two image analysis software has successfully identified six cases of hypodiploid aneuploidy out of a total of 78 blocks examined. In most cases the initial DNA index of the flow analysis histogram (diploid) was less than 1.2, showing that even in the presence of relatively high CVs (>5%), two closely residing peaks or a single bifid peak on flow cytometry is indicative of the presence of aneuploidy of either the hypo or hyperdiploid variety. Present study thus proved that such aneuploid populations could only be confirmed by using image

analysis, where the limits of 0.8-1.2 were considered appropriate for the assignment of diploidy. Overall the study provides confirmatory evidence that image analysis could more sensitively detects aneuploid and tetraploid tumors.

5.3. Prevalence of the state of DNA ploidy status in OSCC

Among the techniques that are applicable for the study of histologic sections of tumours, ploidy analysis, particularly the detection of aneuploidy, is one of the most sensitive and effective indicators of malignant transformation (Brandizzi et al., 2009). Some authors reported that DNA diploidy may be seen in both benign and malignant lesions, but aneuploidy is mainly seen in malignant lesion (Martin et al., 1994; Pinto et al., 1999).

In our OSCC samples, the rate of aneuploidy was around 96.2%, which was comparatively higher and our study was in agreement with the results of other studies (Grässel-Pietrusky and Hornstein, 1982; Staibano et al., 1998; Abou-Elhamd and Habib, 2006). Previous reports on OSCC samples also reported a varying range of aneuploidy levels ranging from 30% (Farrar et al., 1989); 41.7% (Garib et al., 2002); 68% Baretton et al (1995) and 76% (Feichter et al., 1987). In fact the study by Staibano et al. (1998) has found out 100% aneuploids. In another study Raybaud et al (2000) have reported that 50% of the tumours were non-diploid; however, they also observed that the non-diploid cancers were ranging from 17.2% to 87% in cases. Moreover, Francia et al (1997) have noticed that aneuploidy was statistically more frequent in anaplastic carcinoma than in differentiated lesions. Aneuploidy of OSCC is an aggressive malignant tumour and the degree of aneuploidy could reflect the aggressiveness of the tumour as evidence in the present study which come in concordant with Brandizzi et al (2009).

However, results concerning the prognostic importance of DNA ploidy have been contradictory. Ensley and Maciorowoski (1994) showed that DNA ploidy has been suggested as an important predictor for the natural history and prognosis in HNSCC. Struikmans et al. (1998) showed that DNA aneuploidy and DNA tetraploidy have more activity and higher probability in producing local tumour recurrent, and the probability of local recurrence was decreased with diploid tumour. Struikmans et al. (1998) further confirmed the impaired prognosis in DNA aneuploidy tumours and this impaired prognosis was largely ascribed to DNA tetraploidy. Intensive tumour growth was observed in aneuploids than in diploid tumour. Nakada et al. (1999) observed that tumour growth was observed in all patients with DNA aneuploidy and has suggested that it may be useful in characterizing metastatic thyroid carcinoma. But according to our results it could be proved that DNA ploidy level could be used as a prognostic marker.

5.4. Comparison of the state of DNA ploidy between the tumour and its margin

We classified the margin of the tumour as clear margin (5mm and more) and close (1-5mm). But involved margin was not included in this study. The comparison of the DNA ploidy status of the tumour and surrounding margin of the tumor was done using Chi square and McNemar test. The results showed high statistical significance (P value <0.001) between DNA ploidy of the tumour and the margin surrounding it and the null hypothesis as suggested in our study was thus rejected.

Current study showed that diploid normal marginal ploidy status was 84.3% while the aneuploid abnormal margin was showing only 15.7%. We have found that the tumour diploid was highly associated with the diploid margin. The aneuploid margin was highly associated with aneuploid tumour despite the diploid margin were also associated with

the aneuploid tumour. Nevertheless using large sample size would be able to reject the null hypothesis with clinical significance of ploidy status. The resection margins which were included in the study, both in the surface mucosa (at the edge of the tumour) and the submucosal (all around the tumour defect) were in complete agreement with studies of Woolgar (2007).

Surgical margins are frequently mentioned by authors reporting the outcome of OSCC, but always there were limitations with proper definitions, understanding of different tumour site, anatomical restrictions, biological characteristics and extent of surgery all of which have influenced the adequacy of the resection (Batsakis, 1999). The present study has tried to rectify these limitations by incorporating all the details. The study has shown that close (<5 mm) margins infer a poor prognosis in terms of both local recurrence and overall survival. Vikram et al. (1984) also reported similar observation where the presence of tumour within 5 mm of the surgical margin was conferred as poorer prognosis for the patient. However, in that study there was no reason for the use of 5 mm to define adequate resection was given. In another study Sutton et al. (2003) showed close margins were strongly associated with histological indicators of aggressive disease behavior. They suggested that close margins could be regarded as an important indicator for aggressive disease. This suggestion was found more or less to be relevant in our study because the prevalence of DNA aneuploidy of tumours were showing a level of 96.2%. Since the aneuploid status was the most aggressive type of DNA ploidy classification of malignancies, we also strongly recommend that close margins could be utilized as one of the best indicator for aggressive diseases. Current study also showed that even when the surgical margins were diagnosed as tumor free through histopathological analysis, with image analysis it was verified that clear margin to be aneuploid margin, which was in concordant with studies of Vikram (1984).

Patients were classified as having risk of disease recurrence based on the pTNM, margin status, and positive nodal disease. Patients staged as pT1-2 with clear margins and pN0 were considered at low risk for recurrence as according to the reports of Brown et al. (2007). The primary aim of any surgeon in treating OSCC is to achieve adequate ablation and in order to attain this, rim of clinically normal tissues has to be included around the tumour. Larger tumours and tumours in certain anatomical sites appear more reliable to close margins, which might likely reflects the increased difficulty of the surgical resection involved. However, no clinical parameter is associated with the probability of a close or involved margin. So, the surgeon had to wait until the pathologist had examined the resection specimen, and then decide if further treatment should be offered to those patients with close or involved margins (Sutoon et al., 2003). In this regard the clinical application of the DNA ploidy is to obtain a good judgment for the surgeons to have regarding whether the clear or close margin is involved with tumour (aneuploid margin). This will increase the prognosis level of patient to a better state and decrease the rate of recurrence.

5.5. DNA ploidy status of surgical margins and type of surgical margins

In this study most of the clear margin was associated with the diploid margin. It was meant that >5mm from the tumour keep the margin as intact epithelial, but with the presence of , even very small percentage, aneuploid margin in clear margin meant that no use and no clinical significant of this relation. van Houten et al (2004) showed that the resection margins are histologically tumor free and the main principle in tumor surgery is the effort to achieve tumor-free resection margins. Author has evaluated the relationship between locoregional recurrence of the tumor and the status of the resection margins. The prevalence of tumoral infiltration at the resection margins varies from 3.5% to 60% (Ravasz et al., 1991). Slootweg et al (2002) showed approximately 3.9–

32% of patients with HNSCC develop local recurrences despite the presence of microscopically negative margins. This study showed the presence of aneuploid cells in margin could be a serious consequences, this outcome is similar in Böcking et al (2011). The insignificant association of the DNA ploidy with the margin status are in concordance with Bremmer et al (2008)The null hypothesis could not be rejected due to statistically insignificant with the *p=0.756.*

5.6. The state of DNA ploidy of the tumour and the sociodemographic and clinicopathological parameters

The mean age of the patients included in the present study was 59.73 ± 12.26 years with majority of the patients above the age of 50 years (75.6%). In this study, it was found that there were no association between the age of the patients and the DNA ploidy status of the tumours, which could not be rejected the null hypothesis proposed in our study *(P = 0.712*; insignificant). Other researchers reported similar observations on OSCC (Baretton et al., 1995; Garib et al., 2002).

The present study showed a female preponderance with female to male ratio, F:M = 2.25:1. Other studies reported earlier on OSCC also suggested similar pattern of overall female predilection. The studies of Ng et al. (1985) and Siar et al. (1990) showed a F:M ratio of 1.1:1 and 1.4:1 respectively, but, they were not seen as high as in the present study. The female preponderance was seen in Indian populations with OSCC of the buccal mucosa. However, the current study showed no association of DNA ploidy of the OSCC with the gender, where the significance level showed insignificance (*P* = 0.922) and thus the null hypothesis could not be rejected as proposed in the present study. The result from our study was in absolute concordant with the other studies done on OSCC (Baretton et al., 1995; Garib et al., 2002).

Many studies have revealed that the prevalence rate of OSCC was high among Indians (>50%) and low among the Malays and Chinese (Ng et al., 1985; Siar et al., 1990; Ng and siar, 1997). In the current study, the Indian comprised around 71.8% of the cases while the Malays and Chinese contributed only 15.4% and 12.8%; respectively. The highest percentage of Indians was previously reported in the studies of Siar et al. (1990), which was lower than the one reported in our study. The ethnicity also showed lack of association with the DNA ploidy of the tumour as the null hypothesis could not be rejected where the $p = 0.587$ was insignificant.

In this study, betel quid chewing represented the major risk factor among habits (smoking, alcohol, and betel quid chewing). The current study did not show any significant differences between the habits and the DNA ploidy of OSCC. Thus the null hypothesis could not be rejected because there was no statistical significance with all the habits analyzed (Smoking, $P = 0.439$; Alcohol, $P= 0.082$; and betel quid chewing, $P= 0.667$). even our finding showed insignificant in betel quid chewing in association with the DNA ploidy but also found that among the betel chewing consumer 96.7% are associated with the aneuploid DNA and our findings comes with discordant of Lin et al (2011) which showed a high association between the habits and oral cancer.

The association of DNA ploidy and the pTNM stage of the tumour investigated found that the diploid DNA was associated with tumour in stage I and II. From the results it was found that majority of the aneuploid tumours were associated with stage III and IV. In this study, association between DNA ploidy of surgical excision samples and pathological TNM, and lymph node metastasis were showing no significant association, which was in agreement with results of Garib et al. (2002). Although there were no associations between the pTNM and DNA ploidy, it was observed that 59.6% of the

aneuploid level of the tumour were in stage 3 and 4. Previously, Melchiorri et al. (1969) suggested that lymph node status could be used as a powerful prognostic indicator. However, Garib et al. (2002) contradicted the results of Melchiorri et al. (1969). Garib et al. (2002) observed that there was lack of association between DNA ploidy and lymph node status. Our study also revealed similar observations as that of Garib et al. (2002) where there is no association between lymph node status and DNA ploidy status. Thus the null hypothesis was not able be rejected due to insignificance in statistical results, where the pTNM showed the P-value was 0.386 and lymph node metastasis showed a P-value of 0.891.

In this study, there was high significant difference between the histo-pathologic classifications with the DNA ploidy level of the tumour, The histologic grade of differentiation was not predictive despite its good association with DNA ploidy. These results were however, in disagreement with results of the previous studies (Melchiorri et al., 1996; Garib et al., 2002). The null hypothesis could be rejected where the $P = 0.025$ was significant through that the aneuploid DNA was associated with the poor and moderate classifications. Further, the findings of the present study agree with Struikmans et al. (1998), where we demonstrated that DNA tetraploidy had additional prognostic significant in tumour staging. Staibano et al. (1998) reported that DNA content is a useful parameter in evaluation of OSCC of the head and neck but not correlated to the clinical stage of the disease. But, Hemmer et al. (1997) reported an excellent prognosis of patients with diploid primary oral SCC.

Many authors were in discordance with the present results, suggesting that there are no association between aneuploidy in OSCC and clinical factors (Ensley et al., 1989; Baretton et al., 1995). In yet other studies, DNA aneuploidy has been associated with

moderate and poor differentiation (Tytor et al., 1990; Balsara et al., 1994); advanced stage (Kearsley et al., 1991) and larger tumors (Tytor et al., 1990; Balsara et al., 1994). present results come with agreement with studies of Baretton et al. (1995) and Raybaud et al. (2000) where they reported that there were no statistically significant differences between DNA ploidy and factors like age, sex and tumour stage.

The pattern of invasion showed highly statistically significant difference (*p=0.004*) and the aneuploid tumour showed highly association with the pattern of invasion 3 and 4, this results come in disconcordance with the Garib et al. (2002), where the null hypothesis could be rejected through it was statistically proven to be significant.(*P*= 0.004). In this study, the results showed agreement with studies of Baretton et al. (1995) regarding the lack of relation between DNA ploidy and tumour size. Ploidy changes in some preneoplastic lesions of the oral cavity with subsequent malignant formations suggested that ploidy anomalies may even exist before histological modifications were made (Melchiorri et al., 1996).

The present study also found significant difference between the DNA ploidy of the tumour and tumour site, where the significance level was showing P= 0.009. Our results were negative to the results as found by Raybaud et al. (2000). Thus the null hypothesis was rejected. The association of the depth of invasion showed highly significant difference (P = 0.004) in our study the highly associated of DNA aneuploid tumour with the >5mm depth of invasion conclude that the aneuploid DNA associated with the deepest tumour. High associations of DNA aneuploidy with the tumour depth of more than 5mm provide an evidence of local aggressiveness of the malignant area.This result was in concordance with the studies of Fakih et al. (1989) and Yuen et al. (2002) and thus the null hypothesis could be rejected. This would largely enable surgeons to deal

with regions around the neck optimally with patients having higher risk of occult metastasis at the time of first surgery. This would also help to avoid unnecessary surgeries, in patients with lower risk of occult metastasis (Kane et al., 2006).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The conclusions for this study are:

1. The prevalence of aneuploidy in OSCC was 96.2% and diploidy was 3.8%.
2. The null hypotheis that there is no significant association between the tumour DNA ploidy status and ploidy status of the margins was rejected as there was a statistically significant difference ($p<0.001$) between them, where all the diploid tumour were associated with the diploid margins and some aneuploid tumours were associated with aneuploid margins(15.7%).
3. The null hypothesis that there is no significant association between the tumour DNA ploidy status and the pathological types of surgical margins was rejected, as there is a statistically significant difference ($p<0.001$) between them where all close margins were associated with aneuploid tumours and a high percentage of clear margins were associated with aneuploid tumours.
4. The null hypothesis that there is no association between DNA ploidy status of the surgical margins and the pathologic types of surgical margins cannot be rejected as there is no statistically significant difference beween the margin ploidy status and tha pathological types of margin (p-value = 0.75) thus showing a lack of association between these parameters.
5. The null hypotheses that there is no association between the DNA ploidy status of OSCC and the selected sociodemographic and clinicopathologic parameters cannot be rejected for most parameters as there is no statistically significant difference between them. For four parameters namely histopathological classification, pattern of invasion, tumour site and depth of invasion, the null hypothess were rejected, as there were statistically significant differences between them.

Recommendation for further studies on DNA ploidy in OSCC:

- There are indicators for certain parameters showing significance and therefore, a larger sample size is recommended for future studies, to reveal clearly whether the sample size was the critical factors involved with insignificance of various factors.
- Further development of the software in the case of automatic deletion of the overlapping images in which the diploid maybe recorded as tetraploid.
- Since in this study there was a limitation in collecting the information's regarding the patient survival due to short duration of the study, there is a necessity to further analyse the accuracy of using ploidy status as a prognostic indicator. In order to achieve this there is a need to collect the complete information's on patient survival for future studies.
- This study was entirely focused on DNA ploidy in OSCC. Hence it is recommended to evaluate the use of image cytometry for ploidy status in various other types of cancers.
- The additional use of DNA image cytometry is a reasonable tool for the assessment of the resection margins of squamous cell carcinomas. DNA image cytometry could help to find the appropriate treatment option for the patients and thus might improve their prognosis.

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APPENDICES

Appendix 1

Image Cytometry System requirements

a) Hardware requirments

- Illumination: bult-in transmitted Koehler illumination12 V, 100W long-life halogen bulb (pre-centered) 12 V 100 W HAL-L (PHILIPS 7724). Average life time was approximately 2000 hr. when used as directed. Light intensity voltage range: 2Vor less to 12.0 V (continues) with the light intensity preset switch. Rated voltage of external power supply (BX-UCB): 100-120/220-240 V~50/60 Hz, 3.5 A/1.5A. Power consumption (BX-UCB): 50-300 W (variable with number of connected modules).
- Focusing mechanism: Drive system: motorized focusing using a stepping motor and ball screw. Stage height movement by cross roller guides.
  Finest adjustment scale: 1 µm (fine movement sensitivity 1 µm)
  Resolution: 0.01 µm
  Highest stage speed: 3 mm/sec.
- Observation tube: the BX61 come with many type of observation tube one of them are U-SWTR-3 with super wide field trinocula , tube inclination 30^{o}, interpupillary distance adjustment is 50 mm to 76 mm.
- Stage: type U-SVLB-4 common- axis knobs on bottom left. Rectangular ceramic coated. Wire driven stage at size 156 mm (H) x 191 mm (W).
- Movement mechanism: X/Y-axis knobs with adjustable tension. With the movement range 52 mm in Y-axis direction and 76 mm in X-axis direction. The stage is for double slide holder (U-HRDT4).
- Condenser: objective: 2X, 10X, 20X, 40X, 100X.

Appendix 2

DNA QC particles and FCM calibration

Acquiring CEN

1- Set the instrument flow rate to LO, turn the flow cytometer to RUN, mix the CEN sample and install it on the cytometer's Sample Injection Port.

2- Make sure you are in Setup mode and click Acquire in the Acquisition Control window.

3- Make the following two adjustments.

Click Pause and Restart in the Acquisition Control window as many times as needed. All adjustments are made in the Detectors/Amps window.

• Viewing the FL2-A histogram, adjust the FL2-H photomultiplier tube (PMT) Voltage so the CEN singlets are in channel 200 ±5.

• Viewing the FL2-W histogram, adjust only the FL2-W Amp Gain so the CEN singlets are in channel 200 ±5.

The channel number can be approximated by viewing the Mean column of the Histogram Statistics box.

4- Click Pause when done.

5- Click Abort in the Acquisition Control window.

6- Uncheck the Setup box in the Acquisition Control window.

7- Click Acquire.

8- After acquisition is complete, remove the CEN from the Sample Injection Port and place the cytometer in STANDBY.

9- Adjust markers (M) M1 and M2 on the first two peaks, singlet and doublets, of the FL2-A histogram so the peak falls within the respective marker.

10- Calculate the linearity using the mean channel number, located in the Histogram Statistics box, for marker 1 (M1) and mean channel number for marker 2 (M2) using the following formula:

Mean M2 Mean/M1 = linearity

The linearity should be between 1.95 and 2.05. Locate the coefficient of variation (CV) of M1 in the FL2-A Histogram Statistics box. Check to see that the CV is less than or equal to 3.00%.

11- Choose Instrument Settings from the Cytometer menu.

12- Click Save.

13- Type a name for the file, and specify a storage location.

14- Click Save in the directory dialog box.

15- Click done in the Instrument Settings dialog box.

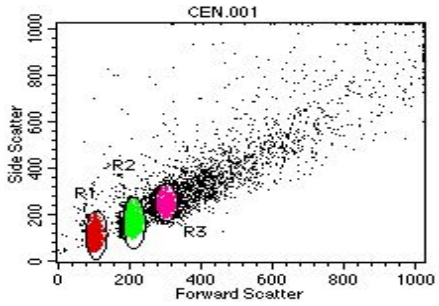

Figure show singlet at R1

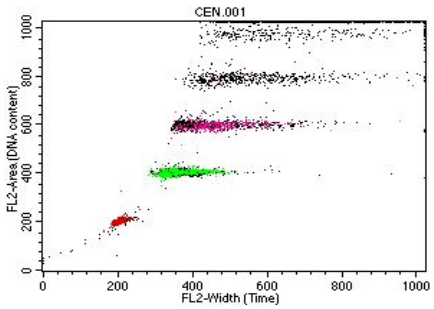

Figure show the singlet at 200 vs 200 on FL2-W vs FL2-A

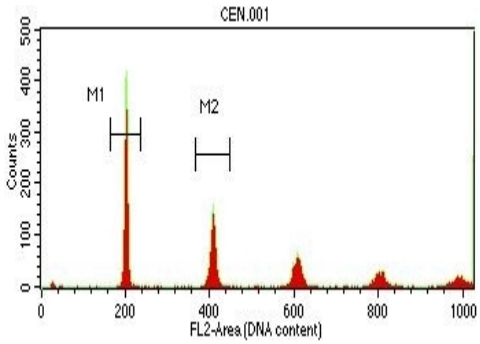

Figure show FL2-A

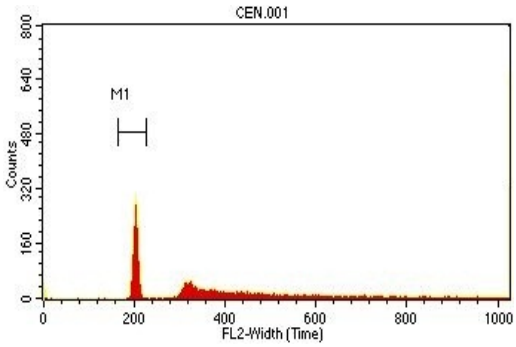

Figure FL2-W singlet on channel 200

Acquiring CTN

To acquire CTN, the plot on page 2 of the DNA Experiment Document will need to be reformatted to an Acquisition -> Analysis plot.

1- Scroll to page 2 of the DNA Experiment Document.

2- Choose Parameter Description from the Acquire menu.

3- Type CTN in the Sample ID box.

4- Close the Parameter Description window.

5- Set the instrument flow rate to LO, turn the cytometer to RUN, and install the CTN sample on the cytometer's Sample Injection Port.

6- Click Acquire. A CTN sample (10,000 events) is collected. View the width versus area dot plot and display all 10,000 events. Verify there is good separation between the G2+M portion of the singlet population and the doublets in FL2-W.

7- After acquisition is complete, remove CTN from the Sample Injection Port and place the cytometer in STANDBY.

8- Save this new Experiment document by choosing Save from the File menu.

9- Type a name for the document and specify a storage location.

10- Click Save.

11- Close the newly saved DNA Experiment Document. The daily quality control of the instrument has been completed. Becton Dickinson recommends running a peripheral blood mononuclear cell (PBMC) sample or other diploid control to further adjust the instrument before acquiring samples. Prepare and stain the PBMCs the same way you would your specimens. For further information, refer to the CELLQuest DNA Experiment Document User's Guide.

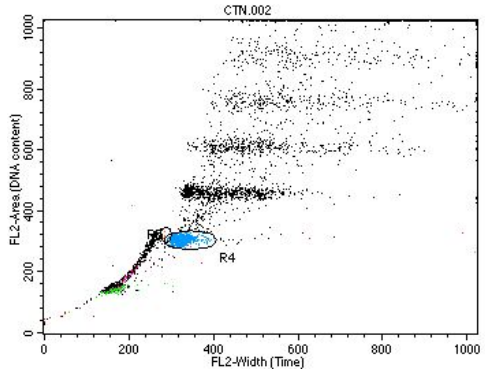

Figure show doublet discrimination in R4

CV Check of 2-μm Beads on FL2-Area

1- Open a new CELLQuest Experiment Document or add a page to the DNA Experiment Document. For further information about Experiment documents refer to the CELL Quest Software User's Guide.

2- Choose Histogram Plot from the Plots menu.

3- Change Plot Source to Acquisition->Analysis.

4- Select FL2-A in the Parameter field and click OK. NOTE: Enlarge the plot for easier viewing.

5- Choose Histogram Stats from the Stats menu.

6- Choose Parameter Description from the Acquire menu. Select Folder, where you will save the data to, and name the file.

7- Close the Parameter Description window.

8- Make sure you are connected to the cytometer and are in setup mode.

9- Set the instrument flow rate to LO, turn the cytometer to RUN, and install the 2-µm beads on the cytometer's Sample Injection Port.

10- Click Acquire.

11- Adjust the FL2 Voltage to position the singlet peak at channel 200 ±5. NOTE: The channel number can be estimated by viewing the Mean channel column in the Histogram Statistics box.

12- Click Pause, Abort, and Setup to remove the check mark.

13- Click Acquire to save the data file to disk.

14- After acquisition is complete, remove the 2-µm beads from the Sample Injection Port and place the cytometer in STANDBY.

15- Place a marker (M1) on the singlet peak and verify that the CV of M1 is less than or equal to 2.3%.

16- This Experiment Document can be saved and printed.

1) Run the control PBMC sample.

a) Adjust the FL2-H voltage (only) to place the first peak at the 200 channel at FL2-A.

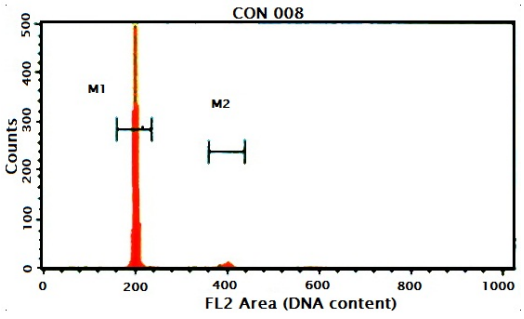

Figure show the PBMC on channel 200

b) Save the instrument setting. And apply it to your OSCC sample.

The events (PBMC) that scanned through the FCM should be not less than 10,000 events (Melchiorri et al., 1996), the linearity which is M2/M1 = (1.95-2.05) and the resolution (CV of M1) = < 3.0%.

Remove the PBMC sample from FCM and using the tube of OSCC with PBMC at ratio 2:1 of OSCC cells to PBMCs for the use of PBMCs as control diploid.

Install the sample (PBMC+OSCC) on FCM machine and be sure that the device run on LOW speed and then press RUN, it is recommended that the samples be run at an acquisition rate of at least 60 events per second.

Adjust the FL2-H voltage (only) to place the 1st peak (diploid peak) at the channel 200 at FL2-A, check the Linearity and Resolution of the Diploid peak and the other peak, save the instrument setting as your experiment setting and print the results obtained.

Appendix 3:

PBMC (Lymphocyte) Separation from Blood by using Ficoll Paque Plus

Follow the catalog manual instruction attached to ficoll paque plus kit (Ficoll-Paque™ PLUS product instruction/protocol sheet, 71-7167-00, AE, 2005-02, GE Healthcare Ltd.):

1. Mix Ficoll-Paque™ PLUS (Ficoll) thoroughly before use by inverting the bottle several times.
2. Add Ficoll to tube (see Table below).
3. Dilute blood 2X with phosphate buffered saline plus 2% fetal bovine serum (PBS+ 2% FBS; Catalog #07905), or other suitable culture medium (Table 1).
4. Layer blood on top of Ficoll being careful to minimize mixing of blood with Ficoll.
5. Centrifuge at room temperature (15 - 25°C) for 30 minutes at 400 *x g* with brake off.
6. Remove and discard upper plasma layer without disturbing the plasma-Ficoll interface.
7. Remove and retain mononuclear cell layer at the plasma-Ficoll interface without disturbing erythrocyte/granulocyte pellet.
8. Wash mononuclear cells once with medium. Note: Ficoll, blood and medium should remain at room temperature (15 - 25°C) for optimum results.

Recommended Volumes and Tube Sizes

Blood (ml)	PBS + 2% FBS (ml)	Ficoll volume (ml)	Tube size (ml)
1	1	1.5	5
2	2	3	14
3	3	3	14
4	4	4	14
5	5	10	50
10	10	15	50
15	15	15	50

Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which have been aggregated

by the Ficoll and, therefore, sediment completely through the Ficoll-Paque PLUS. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of the Ficoll-Paque PLUS solution attain a density great enough to migrate through the Ficoll-Paque PLUS layer (Figure 1)

Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque PLUS with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to short washing steps with a balanced salt solution to remove any platelets, Ficoll-Paque PLUS and plasma.

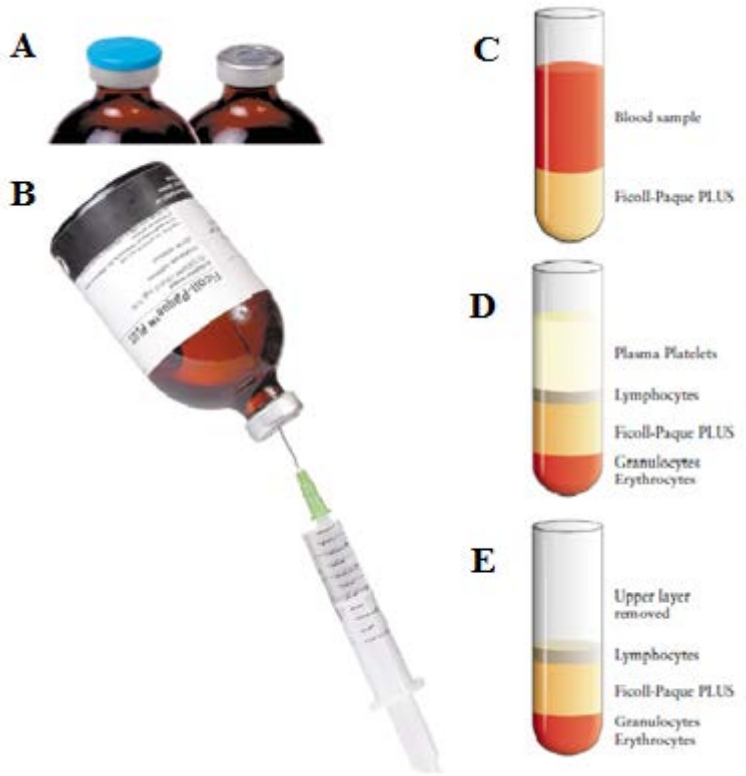

Figure 1 Ficoll paque plus separation

A. Bottle of ficoll paque plus and PBS+2%FBS; B. Drag of ficoll paque plus; C. Blood sample + ficoll paque plus; D. Arrangement of layer after centrifugation; E. Removing the upper layer to drag the lymphocyte.

Appendix 4:

H&E staining

1	Xylene 1	5 Min.	To remove paraffin from sections
2	Xylene 2	4 Min.	To remove paraffin from sections
3	100% Alcohol	3 Min.	To remove xylene
4	95% Alcohol	3 Min.	Approach to water
5	70% Alcohol	3 Min.	Approach to water
6	Running water	3 Min.	Water precedes stains dissolved in water
7	Harris Haematoxylin	10 Min.	To stain nuclei
8	Running water	3 Min.	To rinse off excess stain
9	0.5% Acid alcohol	1-2 Drips	To differentiate; nuclei will retain stain
10	Running water	3 Min.	To rinse off excess acid alcohol
11	2% Sodium Acetate	3-4 Drips	Make stain blue
12	Running water	3 Min.	To rinse off excess sodium acetate
13	80% Alcohol	1 Min.	Partially dehydrates; alcohol precedes stains dissolved in alcohol
14	Eosin	6 Min.	To stai cytoplasm and intercellular substance
15	95% Alcohol	3 Drips	Alcohol destains eosin
16	95% Alcohol	3 Drips	To remove excess eosin
17	100% Alcohol	3 Drips	To dehydrate
18	100% Alcohol	2 Min.	To dehydrate
19	Xylene1	3 Min.	To remove alcohol and clear to clear
20	Xylene2	3 Min.	To clear
21	Xylene3	3 Min.	To clear
22	Mount in D.P.X.		

Appendix 5:

Dewaxing, rehydration and enzyme digestion of FFPE OSCC

A maximum of 5mm x 5mm of tumour FFPE blocks containing ≥60% of tumour and surgical margin tissues were cored out from tumour tissue/surgical margin tissues and re-blocked. Four 50µm (2 sections for flow cytometry (FCM) and 2 sections for image cytometry (ICM)) were cut using a microtom LEICA RM 2245. The sections were placed into 15 ml glass test tube. All the 50µm sections were processed.

all sections 50µm within test tube were dewaxed by putting the samples in an oven at 55^0C for 20 min, the dewaxing and rehydration were carried out through the samples were cleared of paraffin with two changes of xylene for 1 hour at room temperature and rehydrated in a sequence of ethanol concentration 100%, 95%, 70%, and 50% for 30 minutes each at room temperature (Schutte et al., 1985). Wash in distilled water twice.

a) Enzyme digestion for Image Cytometry

Test tube with two sections of 50 µm (for Image Cytometry) were broken down to free the nuclei using protease type XXIV (SIGMA-ALDRICH^{TM} Sdn Bhd.-Malaysia) (Diwakar et al., 2005) by diluting the enzyme with phosphate buffered saline (PBS) pH 7.4 (PBS) to reach the concentration of 0.1% and incubate the samples with 2 ml of 0.1% protease at 37 C^0 for 60 minutes. Vortex at intervals (15 min, 30 min and 45 min) The incubation terminated by adding 2 ml cold ($4C^0$) PBS, after which the tubes were put in ice. Filter through 50µm mesh to remove debris, leaving a solution of nuclei. Wash the nuclei twice with PBS buffer by centrifuge nuclei for 400 g/5min.

b) Enzyme digestion for Flow Cytometry

Test tube with two sections of 50 µm (for flow Cytometry) were broken down to free the nuclei using pepsin enzyme digestion (P-6887, SIGMA-ALDRICH^{TM} Sdn Bhd.-Malaysia); with 5 ml 0.5 % pepsin(5 mg/ml) with PBS and adjust the PH 1.5 at 37 ^oC for 30 minutes, incubate with vortexing the samples. Filtrate the suspension with 50 µm nylon mesh, centrifuge at 400 g for 5 minutes, wash with PBS twice, the cell concentration was adjusted to a maximum of 5 x 10^5cells/ml (Garib et al., 2002).

c) Cell counting for cytospin

Nuclear suspensions for cytocentrifuge (Shandon Cytospin using special cell counting), cytospin at 600 rpm for 8 minutes for the samples of Image Cytometry Analysis.

Count the cells seen in the field. It is not necessary to count every cell, an approximation will do. This count can be used to estimate the number of drops of the cell suspension required for a Shandon Cytospin preparation. To determine the number of cells being used, multiply the number of the cell counted under objective 40X by 38. Divide the number of cells counted into 60. The quotient equals the number of drops that should be added to the Shandon Cytospin sample chamber, through the total volume should not exceed the 0.5 ml capacity of the chamber. This gives the total number of cells applied to the Shandon Cytospin funnel for each drop of suspension used. While technique for estimation of cell number is an approximation only, it does provide an excellent control of Shandon Cytospin preparations.

d) Slides were air dried for 30 minutes and then fixed with 10% buffer formalin overnight.

Appendix 6

PROPIDIUM IODIDE stain for PBMCs control lymphocyte

Manual instruction guide of Cycle Test Plus DNA Reagent Kit

To prepare PBMCs, purify the mononuclear cells using density-gradient centrifugation following the manufacturer's instructions for use of the separation medium

1. Purify mononuclear cells using density-gradient centrifugation; follow the manufacturer's instructions for use of the separation medium.
2. Place the white blood cell suspension into a clean 17 x 100-mm tube.
3. Add 5 mL of Buffer Solution and resuspend the cells by gently vortexing at low speed.
4. Centrifuge for 5 minutes at 300 x *g* at room temperature (20° to 25°C).
5. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet. Resuspend the pellet in 1.5 mL of Buffer Solution by gently vortexing at low speed.
6. Centrifuge for 5 minutes at 300 x *g* at room temperature (20° to 25°C).
7. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet. Resuspend the pellet in 1-mL Buffer Solution by gently vortexing at low speed.
8. Count the cells using standard laboratory methods with a hemacytometer. Adjust the concentration to 1.0 x 10^6 cells/mL with Buffer Solution.
9. Cells are now ready for immediate staining and flow cytometric analysis. Cells may also be frozen for later testing.

Staining Procedure

Powder-free latex gloves should be worn to protect skin from contact with reagents.

NOTE: Use Solution A and B at room temperature (20° to 25°C). Solution C should be kept cold (2° to 8°C) and protected from light.

1. The staining procedure for DNA ploidy analysis requires a test sample of 5.0 x 10^5 cells. An additional sample tube of the specimen mixed or "spiked" with PBMCs should be prepared and used as a control. Use at least a 2:1 ratio of tumour cells to PBMCs.

2. Centrifuge the cell suspensions at 400 x g for 5 minutes at room temperature (20° to 25°C). Carefully decant all the supernatant, and tap off the last drop onto a tissue.
3. Add 250 µL of Solution A (trypsin buffer) to each tube and gently mix by tapping the tube by hand. *Do not vortex.*
4. Allow Solution A to react for 10 minutes at room temperature (20° to 25°C). Do not remove Solution A.
5. Add 200 µL of Solution B (trypsin inhibitor and RNase buffer) to each tube and gently mix by tapping the tube by hand. Do not vortex.
6. Incubate with Solution B for 10 minutes at room temperature (20° to 25°C). Do not remove Solution A and B.
7. Add 200 µL of cold (2° to 8°C) Solution C (propidium iodide stain solution) to each tube. Gently mix as above and incubate for 10 minutes in the dark on ice or in the refrigerator (2° to 8°C).
8. Filter the sample through 50-µm nylon mesh into a labeled 12 x 75-mm tube or use 35-µm cell strainer cap and filter into 12 x 75-mm tube.
9. The samples are now ready to be analyzed on the flow cytometer. Cap or cover the prepared tubes and store at 2° to 8°C in the dark until flow cytometric analysis.
10. Run samples on the flow cytometer within 3 hours after addition of Solution C. After storage, be sure to mix the sample in the tubes by tapping the tube by hand to resuspend the cells.

Appendix 7

PROPIDIUM IODIDE staining for OSCC sample

The CycleTEST PLUS DNA Reagent Kit contains four components:

BECTON DICKINSON Cat. No. 340242

1. Solution A (10 mL): Contains trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons.
2. Solution B (8 mL): Contains trypsin inhibitor and ribonuclease A in citratestabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA.
3. Solution C (8 mL): Contains propidium iodide (PI) and spermine tetrahydrochloride in citrate stabilizing buffer. The PI stoichiometrically binds to the DNA at a final concentration of at least 125 μg/ml.
4. Buffer Solution (3 vials, 50 mL per vial): Contains sodium citrate, sucrose, and dimethyl sulfoxide (DMSO) for the collection and/or freezing of cell suspensions.

SPECIMEN COLLECTION AND PREPARATION-

Cell Suspensions from Solid Tissue

Samples received containing cells already in suspension from tissue culture or body fluids may also be used for DNA analysis.

1. Place cell suspension into a 17 x 100-mm tube.
2. Centrifuge for 5 minutes at 300 x g at room temperature (20° to 25°C).
3. Aspirate the supernatant leaving approximately 50 μL of residual fluid in the tube to avoid disturbing the pellet. Add 1 mL of Buffer Solution and resuspend the cells by gently vortexing at low speed. (repeat two more times)
4. Count the cells by standard laboratory methods using a hemacytometer. Adjust the concentration to 1.0 x 10^6 cells/ml with Buffer Solution.
5. Cells are now ready for immediate staining and flow cytometric analysis. Cells may also be frozen for later testing.

Staining Procedure

Powder-free latex gloves should be worn to protect skin from contact with reagents.

NOTE: Use Solution A and B at room temperature (20° to 25°C). Solution C should be kept cold (2° to 8°C) and protected from light.

1. The staining procedure for DNA ploidy analysis requires a test sample of 5.0 x 10^5 cells. An additional sample tube of the specimen mixed or "spiked" with PBMCs should be prepared and used as a control. Use at least a 2:1 ratio of tumour cells to PBMCs.
2. Centrifuge the cell suspensions at 400 x g for 5 minutes at room temperature (20° to 25°C). Carefully decant all the supernatant, and tap off the last drop onto a tissue.
3. Add 250 µL of Solution A (trypsin buffer) to each tube and gently mix by tapping the tube by hand. *Do not vortex.*
4. Allow Solution A to react for 10 minutes at room temperature (20° to 25°C). Do not remove Solution A.
5. Add 200 µL of Solution B (trypsin inhibitor and RNase buffer) to each tube and gently mix by tapping the tube by hand. Do not vortex.
6. Incubate with Solution B for 10 minutes at room temperature (20° to 25°C). Do not remove Solution A and B.
7. Add 200 µL of cold (2° to 8°C) Solution C (propidium iodide stain solution) to each tube. Gently mix as above and incubate for 10 minutes in the dark on ice or in the refrigerator (2° to 8°C).
8. Filter the sample through 50-µm nylon mesh into a labeled 12 x 75-mm tube or use 35-µm cell strainer cap and filter into 12 x 75-mm tube.
9. The samples are now ready to be analyzed on the flow cytometer. Cap or cover the prepared tubes and store at 2° to 8°C in the dark until flow cytometric analysis.
10. Run samples on the flow cytometer within 3 hours after addition of Solution C. After storage, be sure to mix the sample in the tubes by tapping the tube by hand to resuspend the cells.

Appendix 8:

Blue feulgen staining

Contents:

Blue Feulgen Stain 2 x 500ml

Decolorizer 10 vials

Rinse Reagent 10 vials

Staining Procedure:

1. Hydrate fixed slides in distilled water for 5 minutes.
2. Hydrolyze slides in a coplin jar (plastic) containing 5N HCl for 60 minutes at room temperature (18-25ºC).Seal the jar completely with parafilm during incubation or cap tightly.
3. Place slides in distilled water for 2 minutes to remove excess acid.
4. Place slides in a coplin jar containing Blue Feulgen Stain (decolorized) for 60 minutes. Seal with parafilm or cap tightly. Occasionally a blue band may appear near the top of the staining solution. Prior to removing slides, the jar must be shaken to remove this band.
5. Rinse slides in three changes of distilled water for 2 minutes each. The slides can remain in the third change for up to 10 minutes while preparing for next step.
6. Place slides in three changes of Rinse Solution for 5 minutes each. Seal jars during rinse to reduce liberation of SO2 gas into the laboratory.
7. Rinse slides in three changes of distilled water for 2 minutes each.
8. Dehydrate slides in 70% ethanol for 1 minute.
9. Dehydrate slides in two changes of 95% ethanol for 1 minute each.
10. Dehydrate slides in two changes of 100% ethanol for 1 minute each.
11. Clear slides in two changes of xylene for 1 minute each.
12. Coverslip in medium compatible with clearant used.

Appendix 9

Evaluation of PBMC and FFPE in ICM

The slides consisting of PBMC and FFPE nuclei were placed on the motorized stage of the microscope separately at different times and viewed under the 40X microscope objective. Five areas were selected for viewing for each slide. (**Figure 3.3)**

From each section a minimum of five snapshots/or according to the nuclei/cells present and the images saved for analysis.

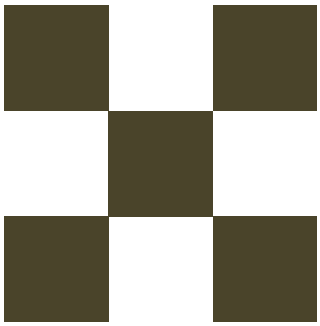

Figure 3.3 Selection of areas for image capturing

The use of another software (commercial software-OTMIAS), which was already calibrated on DNA diploid, was used to compare the results with the Image pro MDA software. OTMIAS software was specifically for DNA ploidy analysis on Feulgen-stained sections or intact cells.

Appendix 10

OTMIAS software analysis

Load the images of PBMC, from the window of OTMIAS, choose step 1 segmentation, choose the nuclei that you want to analyze, use threshold and cutting tool to encircle the area of nucleus, the overlap nuclei, nuclei stick together, or broken nuclei should be ignored. After selection of the nuclei, we classify the nuclei whether they are a reference cells or analyzed cells (internal control or tumour cells), and because from the beginning we load the images of PBMC that were no tumour cells to be selected. **Figure 1**

Put the selected nuclei under the reference category and select not less than two hundred fifty (250) cells (Diwakar et al., 2005) and save this selection for further usage in Image-Pro MDA software to compare the results obtained by the two software for the same selected samples. Analyze the selected nuclei in the next step to be sure that the selection were all in the G0G1 phase. Run the statistics in the next step to check the mean DI (mean DNA index) and CV percentage (coefficient of variant). Save the statistics to compare it with the Image-Pro MDA. Result.

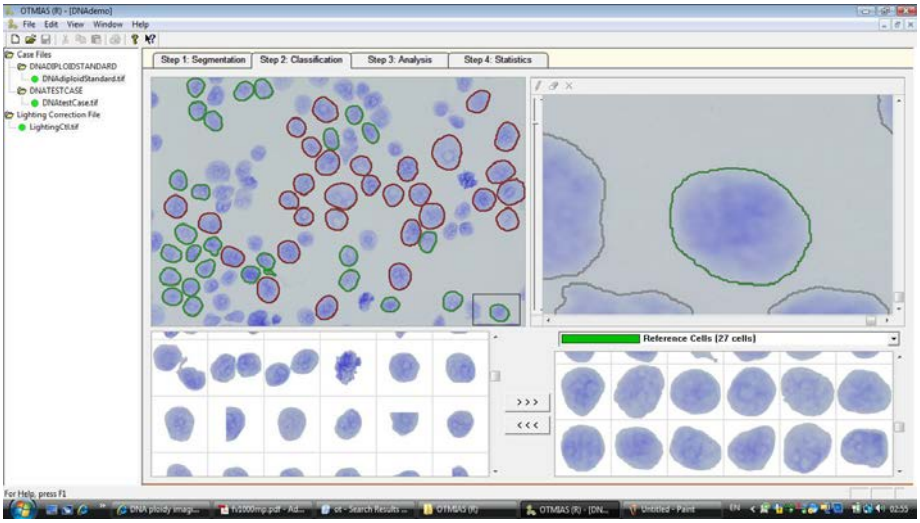

Figure 1 OTMIAS software window

Open new session in OTMIAS window and load the OSCC and margin images, case by case, number the case or rename it, run step 1 (segmentation) to encircle the nuclei of both internal control and tumour or margin cells with a circle line in order to be recognized by the software for analysis, step 2 (classification), select the internal lymphocyte nuclei (internal control) under category of Reference cell which represent

the diploid baseline and should select not less than fifty (50) cells (Raybaud et al., 2000), step 3 (analysis), analyze them to be sure it is all in G0G1 phase. Usually green circle to differentiate it from the other cell selection.

Then select the target (OSCC) nuclei (well circle the nuclei boundaries during segmentation step) which are usually done with red circle, select not less than two hundred fifty (250) nuclei (Raybaud et al., 2000). Analyze them to know the ploidy level of them, the software automatically did the comparison of IOD between the OSCC and internal control lymphocyte nuclei, step 4 (statistics), record and save the result. Repeat the mentioned above and do the same thing with the margin of the tumour.

Appendix 11

Set up the Image Cytometry System

Capturing image using the high resolution camera installed with the software (Image-Pro MDA). At first, power on the microscope and the camera, from the microscope choose the specific level that installed for DNA ploidy and cell analysis, install the slide on the motorized table and set the objective on power 40X. Run the software (Image-Pro MDA) from the computer, select 'image pro' from the menu bar, correct the luminance, brightness and contrast, select to capture image in high resolution (1024x768 (Bin 2)), select the criteria for DNA ploid capture imaging which was developed specifically for this study and save the updating to file to use it next time.

It is important that the images must have the same criteria during image capturing so that to exclude the differences between the images.

1. Set up the filter for the blue feulgen (thionine 590±5 nm).
2. Correct the illumination (Köhler illumination), it acts to generate an extremely even illumination of the sample and ensures that an image of the illumination source (a halogen lamp filament) is not visible in the resulting image.
3. Improve resolution and sample contrast. Adjust the light source for suitable brightness through the using of 40X objective, the diaphragm and condenser lens should be opened, the distance between the ocular lens should be fitted to the operator eye, the high resolution CCD (Q IMAGING Micro Publisher 3.3 camera) (charge-coupled device) and a high resolution LCD (computer screen) should be installed correctly.
4. The luminance, white balance, brightness, contrast, resolution, back ground correction and colour threshold should be fixed for all captured and analyzed images for this study.
5. Nuclei to be measure had to have clear boundaries, any overlap, cut; unclear nucleus had to be ignored.
6. Shading, background per nucleus and stray light could be corrected by software but keep all the values of the mentioned fixed for all captured images.
7. Check the linearity of the IOD values in (2C, 4C, 8C), by: linearity = mean 4C/ mean 2C.

Appendix 12

Image-pro MDA analysis

Image pro MDA was developed specifically for this study, run the software and choose the DNA measurement tool from the menu bar, it contain DNA measurement of reference cell and DNA measurement of test cells. Select “Load” from the DNA ploidy measurement tool, loads the PBMC images, scans the nuclei present in the image and selects the same two hundred and fifty (250) cells that analyzed in OTMIAS software (Diwakar et al., 2005).

For the software calibration on Diploid baseline, select the reference DNA measurement, then determine the minimum and maximum roundness (0.8-1.5), nuclei with circularities of less than 1.5 were accepted for analysis; the circularity of a circle is 1 (Smith et al. 1996), minimum and maximum IOD ($-1x10^4$ – $1x10^8$), and the higher threshold (200) which represent the colour threshold of red, green, and blue (RGB), and from the segmentation window correct the Blue colour because the blue is main colour and the differences in blue condensation refer to differences in chromatin absorption of Blue Feulgen stain, in the other word the IOD condensation. By closing the segmentation window, the analysis was done and appears on screen.

For each nuclei, the minimum, maximum, range, mean, standard deviation of the area, roundness, and IOD. Figure 1

Save the results of IOD of the PBMC for further comparison with the results obtain through OTMIAS software.

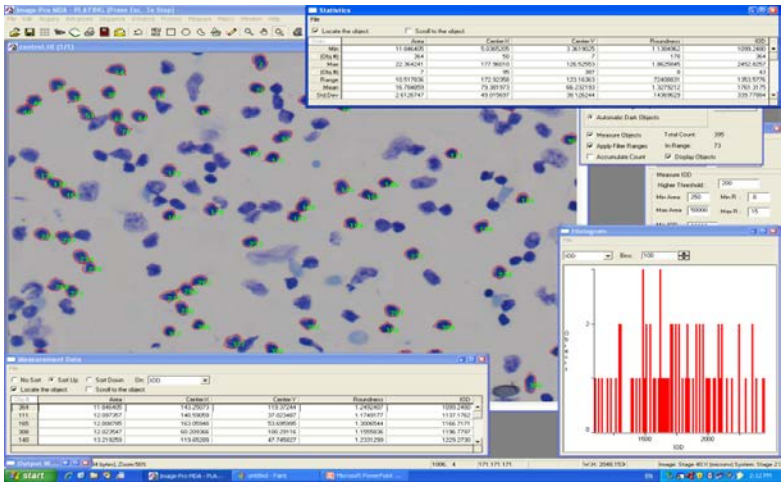

Figure 1 Image-pro MDA

Replace the images of PBMCs and load the images of OSCCs, open DNA ploidy measurement from the menu bar of the software, select the higher threshold at 200, the minimum and maximum roundness (0.8-1.5), minimum and maximum area (250-∞), minimum and maximum IOD ($-1x10^4$ – $1x10^8$), and from the segmentation window correct the blue colour if it was needed and close the window of segmentation, the analysis of each nuclei in which the minimum, maximum, range, mean, standard deviation of the area, roundness, and IOD.

Save the results of IOD of the OSCC for further comparison with the results obtain through OTMIAS software. Run the margin slides using Image-Pro MDA software by following the same steps.