CHAPTER 2: LITERATURE REVIEW

Like many other lifestyle diseases such as coronary heart disease and lung carcinoma, oral squamous cell carcinoma (OSCC) can be very debilitating physically and psychologically and may even cause death. Patients who survived the disease, besides suffering from pain and disfigurement, also loses the ability to chew, swallow, and speak (Chandu et al., 2005; Hassanein et al., 2005; Klug et al., 2002) and these survivors are left feeling anxious and depressed from these losses (Hassanein et al., 2005; Hammerlid et al., 1997).

2.1 Epidemiology

A) Global scenario

The World Health Organization (WHO) released an estimated annual incidence of 418,000 for mouth and oropharynx cancers worldwide in 2002 with a high incidence recorded in the South East Asia region (185,000) as compared to other regions in the world. This significant geographical variation was also highlighted by Johnson (1991) and Moore et al. (2000).

For years, OSCC had been recognized as a disease of old age commonly occurring in the 5^{th} and 6^{th} decade of life. However, recent reports have indicated an emerging trend of this disease occurring in younger patients. Retrospective studies from several countries (Chitapanarux et al., 2005; Martin-Granizo et al., 1997; Mathew lype et al., 2004; Chen et al., 1999; Hindle et al., 1996) revealed the trend even though each used a different cut off age for their studies ranging from 35 to 45 years. Chen et al. (1999), in particular, revealed a shift in the peak age from the 5^{th} decade to the 4^{th} decade from 1985 to 1996. Hindle et

al. (1996), also reported a pronounced increase in incidence rate especially in younger male (35-64 years) from 1982-1986 as compared to from 1962-66.

Discrepancy in gender distribution of the disease was also noted. Men with oral cancer were twice as often as the women in the industrialized countries (Johnson, 1991; Johnson and Warnakulasuriya, 1993). However, Vecchia et al. (2004) noticed a steady and upwards trend in women especially in the European countries.

B) Malaysian scenario

Malaysia, situated in the Southeast Asia, would, at the first impression, be considered as having high prevalence of the disease. However, a national epidemiological survey of oral mucosal lesions in 1993/94 revealed that out of the 11697 subjects examined, only 5 (0.04%) had oral cancer (Zain et al., 1997) and the number did not increase much as compared to another survey in 1973/74 (MOH, 1978). This slight increase in the prevalence in 1993/94 could be due to the inclusion of two other states in Malaysia, i.e. Sabah and Sarawak which were not included in the previous 1973/74 study (Zain and Ghazali, 2001). Despite the considerably low prevalence, oral cancer accounted for 7.1% of cancer deaths in this country (Wan Othman WMN, 2006).

Lim et al. (2002) of the National Cancer Registry reported the incidence of oral cancer to be 1.6 (tongue), and 1.4 (mouth) per 100,000 in Penisular Malaysia as compared to 3.1 by Hirayama (1966). Hirayama (1966) also noted, like in the West (McCartan et al., 2005), the regional variation in the incidence rate, and reported to be highest in the state of Selangor (8.2 per 100,000). About 40 years later, Hamdan et al. (2003) reported that age specific incidence of oral cancer (incidence rate adjusted to world population) was highest

in Negeri Sembilan (1.60) as compared to other states like Perak (0.74), Sarawak (0.66) and Terengganu (0.48).

OSCC in Malaysia usually occurs in the older age groups, i.e. in the 5^{th} and 6^{th} decade of life (Siar et al., 1990; Ng et al., 1985; Zain et al., 1997). However, these studies had also recorded some cases in the younger age groups, i.e. in the 3^{rd} and 4^{th} decade.

Malaysian society is made up of various ethnic groups. The three main ethnic groups are the Malay, Chinese and Indian in the Penisular Malaysia with a few indigenous groups of people in Sabah and Sarawak. Prior to Zain et al.'s report (1997), no data was available regarding the situation of oral cancer in the east Malaysia (Sabah and Sarawak). It was during the nationwide survey of mucosal lesions that the indigenous people of Sabah and Sarawak were identified as a high risk group along with the Indian (Siar et al., 1990; Ng et al., 1985). The same study revealed that women was the most affected, however it is cautionary to note that the working male was not available during the survey time (Zain et al. 1997). Other studies supported the notion, and gender distribution appeared to depend on ethnicity differences. Two separate studies (Siar et al., 1990; Ng et al., 1985) reported M: F ratio of 1:1.4 for the Indian and 2:1 for the Chinese whilst the other reported the ratio of 1:2.14 for the Indian and 2.2:1 for the Chinese respectively while it was 1:1 for the Malay from both the studies.

2.2 Etiological factors

Etiological factors for OSCC are multifactorial. Tobacco, alcohol and quid chewing remain the important causative trio. Other studies have also implicated infections, genetic predispositions, dental-related conditions and nutrition in oral carcinogenesis. The interplay of these factors finally disturbs the cellular turnover of oral mucosa and gives rise to full manifestation of OSCC with varying severities (Patridge, 2000).

2.2.1 Risk Habits

A) Tobacco

Tobacco is undoubtedly the most potent toxin to the human body. Its extensive devastating effects on almost every part of the human body either physically or psychologically are highlighted by the World Health Organization in its publication "The tobacco health toll" (WHO, 2005). Nicotine addiction is the main reason for prolonged usage of tobacco despites all the well known adverse effects (Warnakulasuriya et al., 2005).

Tobacco is used in two main ways; smoking and smokeless, i.e chewing or sniffing tobacco. It is estimated that 44.5 million adults in the USA (CDC, 2005) are current cigarette smokers and an estimate of 1.3 billion people worldwide are cigarette smokers (WHO, 2004). The genotoxicity of tobacco smoke had been reviewed extensively by DeMarini (2004). While the ability of smokeless tobacco in delivering its nicotine is unquestionable (Ayo-Yusuf et al., 2004; Levy et al., 2004; Rodu and Jansson, 2004), its carcinogenic risk is under intense debate even though its products are evaluated to be carcinogenic (IARC, 1998). Either way, significant alterations due to tobacco exposure had been documented at cellular and genetic levels for both habits not only in the oral

cavity (van Oijen et al., 1998; Greer et al., 1983; Hsieh et al., 2001) but in the lung and bladder as well (Hainault and Pfeifer, 2001; LaRue et al., 2000).

While nicotine stimulates and gives a sense of well being (Goodsell, 2004), its derivatives are potent carcinogens. This group of substances is better known as the tobacco specific nitrosamines (TSN) and they are nitrosation derivatives of the nicotine and other amines like nornicotine, anabasine and anatabine that are found in the tobacco. These derivatives are: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-4-(3-pyridyl)-butanol (NNA), N-nitrosononicortine (NNN), N-nitrosoanabasine (NAB) and nitrosoabatabine (NAT). (Hecht and Hoffmann, 1988).

The metabolic activation of these substances to cause DNA damage had been extensively experimented and reviewed (Hecht 1998; Trushin et al., 1994; Pulling et al., 2001; Pressentin et al., 1999; Castonguay et al., 1983)

B) Alcohol

There is no doubt of the synergistic effects of alcohol consumption and tobacco smoking in increasing the risk of OSCC (Mashberg et al., 1993; Ko et al., 1995). Fransceschi et al. (1999) had also demonstrated the increased risk of OSCC with an increase in alcohol consumption if the level of smoking remained constant. This trend was further supported by Hindle et al. (2000), Petti and Scully (2005) and Altieri et al. (2004) and the risk has been demonstrated to be dose dependent (Schildt et al., 1998; Franceschi et al., 2000). The role of different types of alcoholic beverages in OSCC remained controversial (Schildt et al., 1998; Altieri et al., 2004). However, Altieri et al. (2004) concluded that despite the controversy, ethanol is the main component that contributes to the increased risk. Besides

enlarging the size of basal cell nuclei, altering the stratification and increasing percentage of cells in the S-phase at cellular level in the oral mucosa (Maier et al., 1994), significant chromosomal translocations had been shown in chronic alcoholics as well, as compared to the controls (Burim et al., 2004). It was also noted in the same report that the DNA damage persisted even after cessation which coincides with the persistence of increased risk as observed by Franceschi et al. (2000).

C) Quid chewing

Quid chewing is a habit predominantly seen as an eastern culture. High prevalence was recorded in countries like India (Balaram et al., 2002), Pakistan (Mahazir et al., 2006), Taiwan (Ko et al., 1995) and Cambodia (Reichart et al., 1996) Even in the western world, this habit is more commonly practiced by the migrant communities from the eastern countries. (Pickwell et al., 1994)

A number of substances are included in the quid and this had created confusion when reporting oral mucosal lesions associated with its use. Hence, Zain et al. (1999) proposed standardization in reporting quid chewing habit, where quid is defined as "a substance or mixture of substances placed in the mouth or chewed and remaining in contact with the mucosa, usually containing one or both of the two basic ingredients, tobacco and/or areca nut in raw or any manufactured or processed form." Also highlighted was the term "smokeless tobacco" which should be avoided as "quid" can be applied to tobacco products when they are not chewed but placed in the mouth or oral mucosa and remain in contact there.

Three main categories of quid are defined by the specific ingredients included and they are:

1. Areca quid – quid with areca nut but without tobacco products
2. Tobacco quid – quid with tobacco products but without areca nut
3. Tobacco and areca quid – quid with areca as well as tobacco product

Usually a piece of betel leaf is incorporated with the above mentioned substances and it is known as the “betel quid” and other substances should be specified. (Warnakulasuriya, 1997). There can be a variety of ingredients included in the quid such as slaked lime and local spices (Thomas and MacLennan, 1992; Zain et al., 1999)

Histological changes associated with quid chewing have been reported and features included were epithelial atrophy with marked reduction of the rete pegs, hyperortho and/or hyperparakeratosis with subepithelial edema and marked inflammatory changes. Transmission and scanning electron microscopy further revealed cytoplasmic projections of the basal cells into the subepithelial stroma, gaps in the basement membrane with unusual miccrovilli on the cell surfaces, and subepithelial connective tissue was characterized by dense bundles of collagen fibers. (Reichart et al., 1984). Sundqvist and Grafstrom (1992) had also demonstrated that areca nut extract prevented buccal mucosal cells from terminal differentiation and disturbed its cell morphology. Increased DNA single strand break and inhibition of its repair were also noted. Dave et al. (1992) further supported the finding by revealing an increased in the micronucleated cells in the exfoliated cells from the buccal mucosa of quid chewers. Chen et al. (1999) reported safrole-like DNA adducts both in oral tissues from oral cancer patients and *in vitro.*

D) The Trio – tobacco, alcohol and quid

It is interesting to note that the use of the trio has its root in addiction. All were known to produce a more relaxing mood in its users. It is, however, appalling to note the 123-fold increase in incidence when the trio was used together (Ko, et al., 1995) and the increased risk from the combination of any of the two habits (Wen et al., 2005). The presence of N nitrosamines in the substances played a role in oral carcinogenesis by disturbing cellular differentiation and causing DNA damage as discussed above, it increased the probability of cancer initiation because, after all, carcinogenesis is a probabilistic event (Cohen and Ellwein, 1991).

2.2.2 Infections

A) Viruses

Human papilloma virus (HPV) was first suspected to be involved in oral carcinogenesis in 1983 (Syrjänen et al., 1983), several studies had then detected the presence of the virus in OSCC cases. This established cause for uterine cervical and anogenital carcinoma is now a recognized independent risk factor for OSCC (Miller and Johnstone, 2001). The prevalence of HPV infections in OSCC ranged from 15% (Smith et al., 1998) to 100% (Uobe et al., 2001). Two particular “high risk” subtypes are frequently detected in OSCC, i.e. HPV subtype 16 and 18 (Badaraco et al., 2000; Woods et al, 1993; Tsuhako et al., 2000).

HPV 16 and 18 were shown to be able to immortalize human epithelial cells (Band et al., 1990) and HPV 16 was also shown to increase proliferation rate of OSCC cell line and alter its cell adhesion (Kingsley et al., 2006). This ability is attributed to two early viral proteins namely, the E6 and E7, which are important in malignant transformation (zur

Hausen, 2002; Hoffman et al., 2004). The transforming ability of E6 and E7 lies in their abilities to bind to two very important cell cycle control proteins, the p53 and, to a lesser extent, the retinoblastoma protein, pRb, respectively (von Knebel Doeberitz et al., 1994)

Two other viruses were suggested to play a role in oral carcinogenesis, the Epstein Barr Virus (EBV) and human herpes simplex virus (HSV). EBV is strongly associated with Burkitt's lymphoma and nasopharyngeal carcinoma (Nonoyama et al., 1973; Klein et al., 1974). Prevalence of EBV was noted ranging from 15.2% to 76.6% (Kobayashi et al., 1999; Sand et al., 2002; Tsuhako et al., 2000) in OSCC. EBV may share the same transforming mechanism with HPV by binding to p53 and pRb using one of its nuclear proteins, EBV nuclear antigen (EBNA)-5 (Szekely et al., 1993).

The oncogenic role of HSV in OSCC remained inconclusive. Its infection seemed to elevate risk for developing OSCC (Maden et al., 1992). Two subtypes have been implicated, HSV type 1 and 2. Kassim and Daley (1988) reported that 42% of their cases reacted positively with polyclonal antibodies raised against the viral core and capsid proteins of HSV-1. In addition one of the 25 monoclonal antibodies against HSV-infection-related antigens used by Eskinazi and Cantin (1988) also reacted positively to OSCC cell lines and tissues. Others had also found the presence of HSV indirectly in the serum. Shillitoe et al. (1982) found higher titre of neutralizing antibody to HSV-1 in untreated oral cancer patients and in late stage tumours. Shillitoe et al. (1983), using Enzyme-Linked Immunosorbent Assay technique (ELISA) found high levels of IgM antibody towards HSV-1 in untreated oral cancer patients. A range of premalignant and malignant lesions were noted in the female mice cervix treated with HSV type 2 DNA (Anthony et al., 1989) and analysis revealed transforming regions in the HSV DNA,

however its exact mechanism in tunorigenesis remained elusive (Steele and Shillitoe, 1991). Some hypothesized that infection by the HSV conferred a growth advantage for further malignant transformation by other factors (Jones 1995; Hirsch et al., 1984; Park et al., 1986).

B) Bacteria

Syphilis, another sexually transmitted infection, had been implicated in tongue cancer as early as in 1911. Its strong serologic association with tongue cancer was almost suggestive of its causality in a subset of OSCC patients (Dickenson et al., 1995; Meyer and Abbey, 1970; Michalek et al., 1994). However, several issues remained to be resolved; for example, Venereal Disease Research Laboratory (VDRL) test used to detect syphilitic infection could give false positives as a result of infection mononucleosis, hepatitis, tuberculosis or systemic lupus erythematosus and serological false positive could occur in infections with yaws, endemic syphilis (non venereal) and pinta (Dickenson et al., 1995; Meyer and Abbey, 1970). Treatment of syphilis, before the use of antibiotics, was arsenics and heavy metals which were more carcinogenic than the infection itself. Forthcoming evidences regarding the association of these two diseases would be difficult in view of the more effective methods of treating syphilis (Binnie et al., 1983).

It is interesting to note that even normal oral flora might play a role in OSCC. Kurkivuori and colleagues (2006) demonstrated that oral viridans group streptococci was able to metabolize ethanol giving rise to acetaldehyde via its ADH-enzyme activity and *Streptococcus mitis* produced the highest amount of acetaldehyde. Rubinstein and Pedersen (2002) on the other hand found that chewing tobacco sold in the US were frequently

contaminated by the *Bacillus* family of bacteria and demonstrated oral mucosa damage elicited by its exogenous virulence factor(s) via kallikrein/kinin metabolic pathway.

C) Fungal

Oral candidosis is a common manifestation of a weakened immune system in patients with HIV infection. And its frequent association with leukoplakia, a potentially malignant oral lesion and also in epithelial dysplasia (McCullough et al., 2002), had led to its entanglement with oral carcinogenesis. *Candida albicans* strains were shown to be able to metabolize *N*-Benzylmethylamine and nitrite to give rise to *N*-nitrosobezylmethylamine (NBMA). Strains with high nitrosation potential were generally isolated from lesion with more advanced precancerous changes (Krogh et al., 1987). Generation of NBMA in close promixity and possible transportation to deeper layer of the epithelium by its branching mycelium could result in oral tumorigenesis (Hsia et al., 1981; Krogh et al., 1987).

The evidences discussed above suggested a possible sexual transmission which had prompted investigations into the relationship between sexual habits and risk of OSCC. Talamini et al. (2000) and Schwart et al. (1998) found no relation of oral sex in increasing the risk of OSCC; however, it was found that the risk increased with the number of lifetime partners in the latter study.

2.2.3 Genetic Predispositions

In March 2001, Prime et al. published a review on the role of inherited cancer syndromes and their association with OSCC. It was suggested in the review that Li-Fraumeni syndrome (LFS) might predispose a person to OSCC. The idea was quickly refuted by Patrikidou et al., in June 2001 that the suggestion by Prime et al. (2001) was misleading

and that there was no substantial evidence in the literature to associate the syndrome with OSCC. However, both reached the conclusion that the on going evaluation of malignancies in LFS patients is important. Genetic predisposition to cancer in other inherited cancer syndromes are more clear-cut for example, Xeroderma Pigmentosum (XP) with increased risk of basal and squamous cell carcinoma in skin (Sancar, 1996, Chidzonga, 2005).

Another way whereby genetic constitution might play a role in carcinogenesis is in the area of carcinogen metabolism. As was mentioned in 2.2.1 (A), carcinogens had to be activated enzymatically to generate reactive compounds that would damage the DNA. Enzymes that metabolize these carcinogens are termed xenobiotic-metabolising enzymes (XMEs) and the important ones in oral carcinogenesis are alcohol dehydrogenase (ADHs), cytochrome p450 (CYP) and glutathione-S-transferase (GST) and each has its own polymorphisms with varying metabolizing efficacies which may influence the risk of OSCC (Scully et al., 2000). Indeed, in two case control studies, both Sreelekha et al. (2001) and Sato et al. (2000) were able to demonstrate increased risk of OSCC in smokers with CYP1A1 and GSTM1 polymorphisms. In other cancers, Firozi et al. (2002) went further and showed that the DNA adducts level in breast cancer were higher in patients with the above mentioned polymorphisms. The presence of these enzymes for example, the GST, had been noted via immunohistochemistry in various human tissues and tumours raising the questions of inter-individual and inter-organ differences in susceptibility after exposure to carcinogenic substances. (Campbell et al., 1991)

2.2.4 Mouthrinse

In 1979 Weaver and colleagues raised concerns regarding the use of mouthwash in increasing the risk of OSCC. The main concern was the alcohol containing mouthwashes

(Winn et al., 1991; Winn et al., 2001) which had led to the development of new alcohol free mouth washes in the market (Witt et al., 2005). The seriousness and enthusiasm of the issue could be judged from few critical reviews by Elmore and Horwitz (1995), Cole et al. (2003) and Carretero-Pelaez et al. (2004) and the involvement of a major pharmaceutical company, Procter & Gamble Co. These reviews dissected every aspect of the existing studies in the literature from the design of the studies to the results. While Winn et al. (1991) and Winn et al. (2001) gave contradicting results after a decade; these three reviews concluded after rigorous scrutiny that there was no increased risk of OSCC from using alcohol containing mouthwashes.

2.2.5 Dental Factors

A number of studies reported the association of several dental factors such as poor oral hygiene, number of lost and defective teeth and ill fitting dentures with an increased risk of OSCC after adjusting for tobacco and alcohol consumptions (Rosenqiust et al., 2005; Zhang et al., 1990; Lissowska et al., 2003; Marshall et al., 1992) however, Bundgaard et al. (1994) reported otherwise. Velly et al. (1998) went further and investigated the association of these factors in several subsites of OSCC. It was reported that poor oral hygiene due to infrequent toothbrushing was associated with OSCC of the tongue and gum while ill fitting denture was associated with tongue cancers; however, broken teeth were not associated with any specific sites of OSCC.

2.2.6 Diet and nutrition

There have been many concerns regarding the role of diet in cancers. Several studies had looked into the role of vegetables, fruits and micronutrients in oral cancer. Most studies reported an inverse relationship between oral cancer and the consumption of raw

vegetables and fruits (Stefani, et al., 2005; Guneri et al., 2005; Gaudet et al., 2004; Pelucchi et al., 2003; McLaughlin et al., 1988). Toporcov et al. (2004) described the protective effect of fruits intake against oral cancer and suggested that habitual consumption of food high in animal fat and fried food increased the risk. In a report by Negri et al. (2000), among the 17 selected micronutrients studied, protective effects were strongest for carotene, vitamin C, vitamin B6, folic acid, niacin and potassium. Indeed a lower level of several micronutrients such as vitamin B, folate, alpha and beta carotene, lycopene and alpha tocopherol were found in the serum and buccal mucosa cells of chronic smokers (Gabriel et al., 2006; Gabriel et al., 2006). The exact protective mechanism of these micronutrients is not clear at the moment but it could be due to the antioxidative (Stahl and Sies, 2005) and suppression of cell proliferative abilities (Yoshida et al., 2005).

2.2.7 Immunosuppression

The exact mechanism of de novo malignancies after transplantation is still largely unknown. The appearance of malignancies in a wide range of organs including the head and neck was reported to arise ranging from 0.4 yrs to 15 years following transplantation of liver, renal and heart (Catena et al., 2001; Winkelhorst et al., 2001; Haagsma et al, 2001). Hernandez et al. (2003) in particular reported the rapid progression from leukoplakia to oral cancer in just 4 months after liver transplantation. The causative agent remained obscure as the patient was given immunosuppressive regime and prednisolone after the transplantation and was a known chronic smoker and alcohol consumer. There also seemed to be a predilection for squamous cell carcinoma of lower lip in renal transplantation patients (de Visscher et al., 1997)

2.2.8 Others

Other factors involved in increasing the risk of oral cancer included chronic exposure to sunlight particularly, the ultraviolet B (UVB) to SCC of the lip especially the lower lip (de Visccher and van de Waal, 1998; Luna-Ortiz et al., 2004; Chizonga, 2005; Gallangher and Lee, 2006). Several studies in the South America reported the association between mate drinking and upper aerodigestive tract SCC including the tongue. This particular observation could be due to the carcinogenic substances in mate or probably the high temperature when it was consumed (Castellsague et al., 2000; Castelletto et al., 1994; Sewram et al., 2003; Oreggia et al., 1991).

It is not difficult to observe a gene-environment interaction in oral carcinogenesis. Brennan (2002) highlighted the inconsistent and inconclusive results that many laboratory and epidemiological studies reported in identifying the genes involved and their assessment in cancer etiology, and in particular, whether the risk of cancer associated with a particular environmental exposure differs with respect to functionally different polymorphisms of these genes. The commentary also called for larger samples sizes and collaboration among investigators to solve the problems of inconsistencies.

2.3 Oral squamous cell carcinoma – diagnosis, prognosis and survival

Various investigators had emphasized on the importance of early diagnosis, stressing on the role of dental practitioners in detecting the disease (Onizawa et al., 2003) and creating awareness in individuals with high risk habits (Tromp et al., 2005; Kredpon and Sriplung, 2001). Besides these efforts, there is also a rapid development in the tools for detecting the

disease as early as possible such as the use of toluidine blue and exfoliative cytology (Epstein et al., 2002).

Nonetheless, low survival rate of OSCC patients remained as a major concern for many. Leite and Koifman (1997) reported a 5-year survival rate of slightly over 20% with poorer prognosis for disease in stage IV and tongue carcinoma. Carvalho et al. (2004) reviewed a total of 6281 patients from 1953 to 1997 and found an improvement of survival rate from 28.7% (1950s) to 43.2% (1990s) when surgical intervention was incorporated before radiotherapy regime. Woolgar et al. (1999) further supported Leite and Koifman (1997)'s notion and observed an inverse relationship between staging and survival rate. This group also reported a sharp drop in survival rate from 81% with no nodal involvement to 64% with intranodal metastasis and a further rapid decrease to 21% if extracapsular metastasis was found.

Development of the second primary tumours further reduced the survival of the patients. Initially recognized by Slaughter and colleagues in 1953, they observed an incidence of 11.2% of independent multiple tumours in 783 OSCC cases. Microscopically, the group also found multicentric origin for these recurrences and these origins often exhibited abnormal, hyperplastic and atypical epithelium surrounding the tumours in varying distances. Hence they proposed the concept of "field cancerization" whereby multiple areas in the epithelium was preconditioned for malignant change under the perpetual influence of carcinogenic substances. Since then, several groups had questioned if the occurrence of second primary tumours was indeed a result of clonal expansion or arose independently. Various groups examined the microsatellite alterations and found that at least a proportion of the OSCC shared a common origin (Califano et al., 1996, Partridge et

al., 1997, Bedi et al., 1996). Two other alternative theories were hence proposed based on the premise that transforming event was rare and that multiple lesions arose due to widespread migration of transformed cells through 1) saliva (micrometastases) or 2) intraepithelial migration of the progeny of the initially transformed cells (Bedi et al., 1996). However, van Oijen and Slootweg (2000) argued that second primary tumours arose due to the effect of carcinogen-induced field cancerization instead of the alternate theories that were put forth by Bedi and colleagues (1996). They found that multiple remote tumours developed as a result of continuous carcinogenetic influence of alcohol and/or tobacco instead of the migration of tumour cells.

The use of less-than-perfect benchmarks prognostification systems i.e. the tumour-node-metastasis (TNM) system and the histopathological grading had great impact to the prognosis of the patients. The TNM system deals with the anatomic extend of malignant solid tumours. Despite being an imperfect system, it is used widely and remained an important guide to treat and predict outcome (Bettendorf et al., 2004). Recognizing its inherent weaknesses, this system had undergone many revisions. Several modifications were also suggested. Jones et al. (1993) introduced TANIS (T and N integer score). In this modification, the integer values of T and N were added to give score ranging from 1 to 7 as compared to the conventional TNM I, II, III and IV. TANIS was found to perform significantly better in predicting response and survival. Another modification by Hall et al. (1998) combined N1 and N2a into “N limited” and N2b, N2c and N3 into “N extended”. By using this modification on 637 patients and reported their results in term of relative risk, this group found that this simpler system was better in identifying patients with similar survival. A third dimension, the thickness or depth, was suggested to be incorporated in assessing the metastases potential of tumour. O-charoenrat et al. (2003)

showed that 5 mm tumour thickness of tongue was correlated with cervical metastases and that patients with tumour thickness of more than 5 mm had worse overall survival, disease-specific survival and relapse free survival.

The second widely used prognostification system is the histopathological grading. This cornerstone grading system had also seen many modifications in order to improve its prognostic power. The classification based on the proportion of differentiated cells to undifferentiated cells in the Broders classification was found to be lacking in prognosing and assisting in choice of treatment. There is also the problem of subjective interpretation of what is observed under the microscope (Morris, 1994). Before the introduction of a multifactorial malignancy grading by Jakobsson et al. in 1973, there had been many investigators reporting histopathological features that were important in prognosis (Anneroth et al., 1987). Eight features were looked at 1) the structure, 2) differentiation, 3) nuclear polymorphism, 4) mitoses in the tumour cell population and 5) mode of invasion, 6) stage of invasion, 7) vascular invasion and 8) cellular response at the tumour-host interface. Each feature was given 1-4 points and the total points indicated the aggressiveness of the tumour (Jakobsson et al., 1973). Reassessing the validity of this system, Anneroth and Hansen (1984) graded 52 cases of OSCC of the tongue and floor of the mouth. While confirming the improved prognostic power of this multifactorial grading system as compared to the Broders classification, Anneroth and Hansen (1984) also presented several difficulties encountered such as the influence of sectioning in assessing the “pattern” and “mode of invasion”, the different in degree of keratinization in varied sublocations and separating the inflammatory responses due to ulceration from a local immunologic reaction. Modifying the Jakobsson et al.’s (1973) system and using only six parameters, Anneroth et al. (1986) assessed 89 cases of OSCC in the floor of the mouth

and found that the mean total malignancy scoring correlated significantly to clinical staging, frequency of recurrence and survival. Of the many modifications, the introduction of "pattern of invasion" to replace the original "structure" and "mode of invasion" by Crissman et al. (1984) remained an important one. It was found that this histologic variable looking at the cohesiveness of the tumour population was the most important single parameter in predicting survival in 77 oropharynx squamous cell carcinoma. A better 5-year survival rate was observed in tumours with pushing borders and infiltration in large, cohesive cords (60%) than tumour that invaded with small, irregular cords and infiltration as individual cells (37.5%). However, when statistically testing the histological parameters only, frequency of mitoses emerged as an important predictor of survival. Nonetheless, pattern of invasion was still the only parameter that approached significance. It had been realized by many that the use of multifactorial histopathological grading assumed equal importance in all parameters and that it was possible that some parameters were more important than the others (Anneroth & Hansen, 1984; Jakobsson et al., 1973; Crissman et al., 1984).

Another common observation of the OSCC that the tumour exhibited more differentiation with frequent keratinization on the surface than the invasive deeper areas (Jakobsson et al., 1973; Willen et al., 1975) had prompted the introduction of a new histopathological grading by Bryne et al. (1989). Only the most anaplastic areas within the most invasive sites of the tumours were suggested to be graded in this new system. Further modifying the system (by omitting the parameter "stage of invasion" due to insufficient amount of tissues) put forth by Anneroth et al. (1987), the malignancy scores from the most invasive front was found to be superior in prognosis than the Broders' classification (Bryne et al., 1989). Statistically, it was also found that by using biopsy specimens alone, this new

grading system could predict the prognosis for the patients. However, it is of utmost importance to include large and representative biopsies for the grading. It was reported that this new grading system had better reproducibility among its users and that it would get better with calibration (Bryne et al., 1991). Subsequently, several studies had indeed reported the high prognostic value for this system now known as the "Invasive Cell Grading" (ICG). Bryne et al. (1992) reconfirmed that grading of the invasive tumour margin is an independent factor and also demonstrated the increased interobserver agreement calculated by kappa statistics from 0.44 to 0.63 (Bryne et al., 1991). Odell et al. (1994) and Jessen and Boysen (1995) demonstrated likewise results with emphasis on the strong prognostic value of pattern of invasion. Even though with more studies validating the system (Woolgar et al., 1995), there was still a need to further improve the system to be more objective in assessing the aggressiveness of the tumour based on its biological activities (Bryne et al., 1998). Several of these molecular events such as gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and markers of angiogenesis had been studied (Bryne, 1998). This had led to the hypothesis that these activities at the invasive front were representative of the tumour characteristic and better reflect its prognosis (Bryne, 1998). Understanding the biology of OSCC would revolutionalize every aspects of its management and further improve the quality of life for the patients (Takes, 2004). For example, the use of non invasive optical techniques like Elastic Scattering spectroscopy (ESS) and Raman spectroscopy to detect dyplastic and malignant tissues by detecting the deflected wavelength from the different biological makeup of the tissues (Swinson et al., 2005). Furthermore, the detection of p53 mutations in histologically negative margins might predict local tumour recurrence (Brennan et al., 1995) and the use of non surgical treatment such as the gene therapy (Shillitoe, 1998)

2.4 Oral carcinogenesis – cellular and molecular basis

It is now widely accepted that oral cancer arises from an accumulation of genetic errors and followed by clonal expansion. Phenotype manifestation is ultimately an expression of the underlying genetic makeup. Fearon and Vogelstein (1990) had elegantly delineated the distinct underlying genetic damages and its accumulation in colorectal carcinogenesis. This pioneer genetic model had become the paradigm for other human cancers. However, Garcia-Garcia et al. (2005) put forth a provoking thought that tumour may be initiated in the absence of any underlying mutation and is simply a response to external factors. This is seen in the extensive parallel analysis of gene expression using DNA microarrays which showed that a large number of genes in the tumour had increased expression with respect to the corresponding normal tissues. And that morphological and behavioural difference in different tumours may be more closely related to the genetic heritage of the original undisturbed tissue than to the genes newly expressed during carcinogenesis which enables the pathologists to identify neoplasms originating from different tissues.

2.4.1 Cellular basis of oral carcinogenesis

In oral carcinogenesis, the epithelium follows through a series of distinct deranged maturation pattern starting with the classical morphologic progression of intraepithelial neoplasia characterized by pronounced basal cell hyperplasia and irregular basement membrane profile but without cytologic abnormalities. Further development into mild dysplasia includes the previous morphology with cytological abnormalities such as increased cytoplasmic ratio, nuclear hyperchromatinism and irregular nuclear chromatin which is confined to approximately one third of the full thickness of the epithelium. Progression into moderate dysplasia involves half of the thickness of the epithelium with disorganized spinous layer, loss of normal layering or polarity with occasional dyskeratotic

cells. In severe dysplasia, the whole epithelium is involved with evidence of little or no maturation. And the hallmark of an invasive carcinoma is characterized by the escape of cells in the epithelial compartment through the basement membrane. However, it is more important to observe the cytological evidence of dysplasia rather than on the degree of involvement of the epithelial compartment as invasive carcinoma may develop from dysplastic tissues involving less than half of the epithelium.

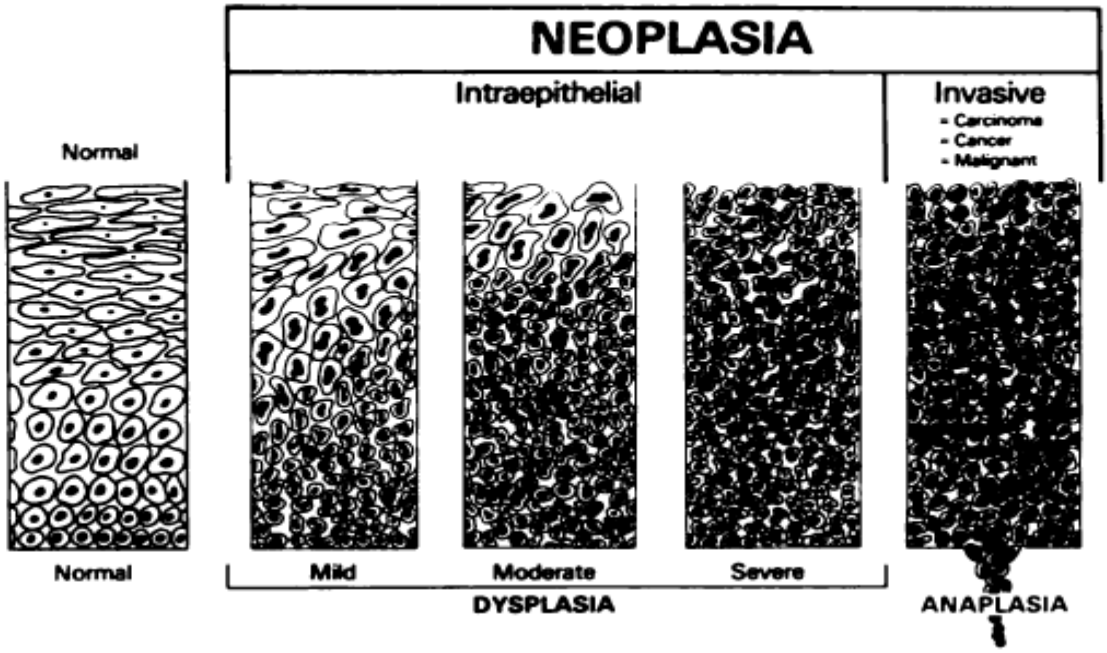

Figure 2.1: Development of intraepithelial neoplasia (dysplasia). Adapted from Boone et al. (1992).

The normal cell population in a given tissue is composed of stem cells, cells committed to differentiation and fully differentiated cells, and in the oral epithelium, these categories are readily visualized with stem cells in the basal cell layer, committed cells in the basal and intermediate layers and fully differentiated cells form the superficial layers (Wright and Alison, 1984).

In order to propagate, genetic errors must occur in cells with the potential to develop into cancer, which in general, are the stem cells. Under normal circumstances, the stem cells divides into stem cell to replace itself and into a cell committed to differentiation to replace fully differentiated cells that have died and have been removed. This replacement process is vital for maintaining the steady-state of a given organ. Rarely, a daughter cell acquires one of the genetic errors required for cancer development since DNA replication is not 100% precise. This cell then enters an intermediate population, so labeled because it has progressed partially along the pathway to full cancer development. Ultimately when the final genetic mistake occurs (and accumulation of previous genetic alterations), this population will produce cells that will give rise to a malignancy. Hence, an agent can increase the development of cancer in two ways: either by specifically damaging the DNA of the cell or by increasing the number of cell divisions, thereby providing greater opportunity for a spontaneous genetic error to occur during normal cellular DNA replication (Cohen and Ellwein, 1991).

In the concept of clonal evolution of cancer, not all progeny of the ill-fated daughter cell which has acquired the genetic error survives, only few mutants that have an additional selective growth advantage expand and become predominant subpopulation within the neoplastic population and demonstrate the characteristics that are recognized as tumour progression (Nowell, 1986). With this, it had led to a much debated concept of tumour heterogeneity.

Tumour heterogeneity seems to be a concept that contradicts the notion that cancer arises from a single cell with altered genes. However, Heppner (1984) strictly confined tumour heterogeneity to cases in which tumour cell differences are believed to be due to difference

in cell lineage, i.e., due to the presence of distinctly different subpopulations capable of breeding true, explained otherwise since tumour appears to undergo developmental and differentiative changes and some of which result from altered gene expression. Hence, in a homogenously looking tumour population, cells with more metastatic potential may exist (Nicolson, 1987). The heterogeneity was probably the reason why Broders classification failed in prognosis as highlighted by Anneroth et al. (1987).

The histological detectable cytologic abnormalities such as increased cytoplasmic ratio, nuclear hyperchromatinism and irregular nuclear chromatin underscores another hallmark of tumour characteristic, genetic instability (Nicolson, 1987, Nowell, 1986). Weber et al. (1998) demonstrated the DNA copy number changes and chromosomal imbalances and suggested clonal evolution in OSCC. Indeed there had been suggestion that six to ten independent genetic events were needed for the development of head and neck SCC (Sidransky, 1995). And in searching for a genetic model for oral carcinogenesis, several chromosomal abnormalities were suggested to be responsible for the stepwise progression of OSCC (Fig 2.2) (Califano et al., 1996).

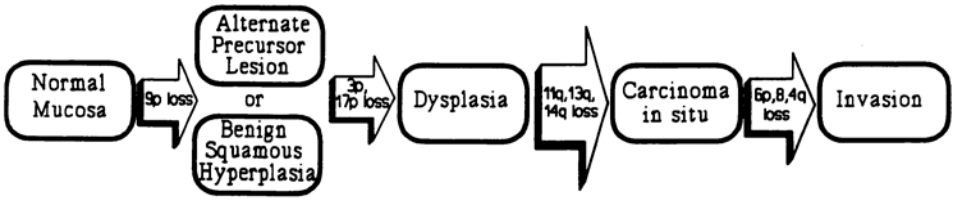

Figure 2.2: Genetic progression model for head and neck cancers. Adapted from Califano et al., 1996.

2.4.2 Molecular event of oral carcinogenesis

Technological advances are revolutionizing the methodologies of cancer research. The use of laser captured microdissection has greatly reduced the problem of tissue heterogeneity and contamination. Other inventions included the use of microarray where a substantial number of genes involved in carcinogenesis can be analyzed simultaneously (Patel et al., 2001; Alevizos et al., 2001). On top of these advances, the application of immunohistochemistry has also contributed to the diagnosis of difficult and equivocal tumours and also provided insight into tumour histopathogenesis (Jordan et al., 2002). Chromosomal abnormalities depicted in Fig 2.2 usually involved genetic loci which are responsible for cell cycle control for example tumour suppressor gene, p53 at chromosome 17p (Voravud et al., 1993).

2.4.2.1 Cell cycle control

The normal cell cycle consists of four phases; the biosynthetic phase (G1), the DNA synthesis (S) and another phase (G2) prior to the final cell division phase, the mitosis (M) as outlined in Fig 2. The progression of the cycle is under complex control. Responding to external and internal stimulus through complex signaling, the nucleus subsequently transcribes particular genes that are needed for the passage through the cell cycle or induction of differentiation (Scully, 1993).

Under normal circumstances, there is a balance in the cell proliferation and cell death. However, in the event of carcinogenesis, the equilibrium is perturbed by three mechanisms; a) an increase in cell production rate, b) a reduced cell loss rate and c) a simultaneous change in both rates (Wright and Alison, 1984).

The cell production rate and loss are governed by two large groups of genes, namely, the oncogenes and tumour suppressor genes, respectively. Oncogenes are genes inappropriately activated from cellular proto-oncogenes and fuels the proliferative activities during tumorigenesis. This inappropriate activation can involve mutational changes in the protein, or constitutive activation, over-expression or failure to turn off expression at the appropriate time (Lewin, 2000). Tumour suppressor genes have the opposite effects. Its actions halt the progression of cell cycle in unfavorable conditions as well as for DNA damage repair and induce apoptosis (cell death) when the DNA damage is beyond repair. Loss of function in these genes promotes tumorigenesis.

2.4.2.2 Measuring proliferative rate

Proliferative indices are the numerical values of the fraction of the cell population occupying any phase of the cell cycle. For examples, the mitotic index is simply the proportion of the cell population in mitosis at any time and the labelling index gives the proportion of cells which are in the S phase (Wright and Alison, 1984)

The mitotic index is simply derived from observation since in most in vivo situation the epithelial tissues are viewed in sections. The labelling index, on the other hand, is more complicated with its use of radioactive nucleotide H^3 -thymidine and autoradiographs. Several methods are derived from the use of these materials:

a) The fraction labeled mitosis (FLM) method
b) The continous labelling method
c) The grain count halving
d) The double labelling method

Besides these, other methods included mircrodentsitometry and flow cytometry. These two techniques relied on the staining of DNA by certain dyes and measurement of the light absorbed by the nucleus at certain wavelength gives the distribution of cells in each phase (Wright and Alison, 1984).

The advantages, disadvantages and the technical problems with each technique had been highlighted (Wright and Alison, 1984) and the final decision on which method to be used would be based on its ease to set up and interpret (Hitchhock, 1991).

Until recently, immunohistological methods of assessing proliferation has gained popularity due to its maintenance of cellular and tissue architecture, the relative simplicity of the methodology and rapidity of results; there is no in vitro or in vivo labeling needed and the use of radioactivity is avoided. One of the most common markers for proliferation is Ki-67 (Hall and Levison, 1990).

A) Ki-67

Developed further from a report of the presence of an autoantibody against the nuclei of acute myeloid leukemia myeloblasts by Klein et al. (1974), Gerdes and colleagues produced a mouse monoclonal antibody and named it, Ki-67. It was reported that this antibody targeted specifically at the nuclear antigen of only proliferating cells but not in resting cells (Gerdes et al., 1983). In the following year, Gerdes et al. (1984) demonstrated the continuous presence of Ki-67 nuclear antigen in S, G2 and M phase but was absent in G0 and suggested the possible reliable role of Ki-67 in evaluating the growth fraction of normal and human cancer cell populations. It was Verheijen et al. (1989) who painstakingly shown that Ki-67, in fact, reacted with antigen in the nucleoli and predominantly localized in the nucleolar cortex and in the dense fibrillar components in

interphase cells. However the group failed to characterize the antigen which was recognized by Ki-67. In another report, Verheijen et al. (1989) went further and demonstrated that the antigen was probably associated with the non-histone proteinaceous structure known as the chromosome scaffold or was an integral part of it. Finally, Gerdes et al. (1992) described the antigen to be a nonhistone protein assembled by polypeptide chains with molecular weight of 345 and 395kd. It was Schonk et al. (1989) and Duchrow et al. (1996) who map the gene enconding the antigen to chromosome 10q.

B) Ki-67 – proliferative cum prognostic marker

In its initial application to several normal human and neoplastic tissues, Ki-67 was found to exhibit parallel staining on proliferating cells as detected by H^3-thymidine method (Gerdes et al., 1983). Applying to a variety of tumors, Sasaki et al. (1988) reconfirmed the parallel relationship between the Ki-67 labeling index and BrdUrd labeling index and concluded that Ki-67 labeling index was an accurate indication of the growth fraction of tumors. In fact Hall et al. (1990) had used it as a benchmark to validate the usefulness of another potential proliferative marker, the PCNA (Proliferating Cell Nuclear Antigen).

However, the prototype Ki-67 antibody had its limitation as it could only be used in frozen tissues since the epitope that it recognized was destroyed in tissue fixation. To solve the problem, several other monoclonal antibodies were raised by using recombinant technology and three of which were designated MIB-1, 2 and 3. It was then demonstrated that these three antibodies were equivalent to the original Ki-67 antibody and that they reacted in the same fashion as the prototype in fresh and formalin-fixed, paraffin-embedded materials (Cattoretti et al., 1992). However, this would only be possible after microwaving the paraffin sections to unmask the antigenic sites as shown by Shi et al.

(1991). Later on, a polyclonal antibody was developed by Key et al. (1993) and it was found that besides its ability to detect Ki-67 antigen, it also facilitated the staining of another monoclonal antibody for double immunostaining method to be feasible. Reynolds et al. (1995) reported a more superior polyclonal anti-Ki-67 antibody raised in sheep instead of rabbits or mouse as previously described and found that this new antibody produced better staining with no cross reactivity. With a few other antibodies available for detection of cell proliferation, Rose et al. (1994) did a study and reported that the most suitable antibodies for routine work was still either the MIB1 or polyclonal Ki-67. A comparison later of equivalent Ki-67 antibodies revealed that MIB-1 was able to detected Ki-67 antigen with the highest sensitivity (Lindboe and Torp, 2002).

Ki-67 antibodies had since become a powerful tool in describing the proliferative states of any tissues, normal or cancerous. Ki-67 labeling index measured the percentage of stained cells in the area of interest, the higher the percentage, the more proliferation in the tissues. Numerous studies had investigated the possible role of this index as a prognostic marker. It had been shown indeed that Ki-67 labeling index might contain significant survival and clinical information in renal cell carcinoma (Delahunt et al., 1995), esophagus squamous cell carcinoma (Youssef et al., 1995) and breast cancer (Veronese et al., 1993). In laryngeal carcinogenesis, it was reported that the Ki-67 expression correlated with increasing severity of the lesion (Krecicki et al., 1999).

In OSCC, Macluskey et al. (1999) also reported an increase in the proliferative index in dysplastic tissues than normal oral mucosa. Other groups demonstrated contradicting results when investigating its prognostic potential. Sittel et al. (1999) reported early relapse in patients with high Ki-67 index as supported by Xie et al. (1999) while Bettendorf and

Herrmann (2002) and Roland et al. (1994) found no prognostic relevance. More recent studies had included assessing the expression at the tumour invasive front. Tumuluri et al. (2002) reported a strong positive relationship between Ki-67 at the tumour invasive front and poorly differentiated tumour. However, both Abdul Jalil (2003) and Lee (2004) did not find any correlation of Ki-67 expression to any of the clinicopathological parameters. The difference might be the smaller sample size in both studies.

2.4.2.3 Tumour suppressor genes (TSGs)

This group of genes represents the "brake" to prevent progression of the cell cycle in unfavourable conditions such as nutrient deprivation, temperature changes or other stresses, nucleotide depletion or damage to the DNA (Kastan, 1997).

Acting in a mendelian recessive manner, a few of the gene members are involved in a number of hereditary cancers as outlined in Table 2.1. The classical example would be Knudson's (1971) "two-hit" mutations in retinoblastoma patients. In the inherited form, the retinoblastoma gene was reduced to homozygosity (i.e loss of heterozygosity) when the remaining surviving wild type allele was inactivated. With this the cell loses its ability to control proliferation (Weinberg, 1992). The most notable tumour suppressor of this group would be the p53 gene. The title "molecule of the year" awarded by the Science magazine in 1993 underscored its pivotal role in human physiology.

Table 2.1. TSGs associated with tumours. Adapted from Scully (1992)

Tumours	Chromosomes involved
1. Familial adenomatous polyposis	6
2. Wilm's tumour	11
3. Retinoblastoma	13
4. Mutiple endocrine neoplasia type 1	11

A) Molecule of the year 1993, the p53.

The intense interest, the burgeoning of literature and the award for this gene was probably due to the fact that p53 is the most commonly mutated gene in many human solid cancers. In an extensive and impressive compilation, Greenblatt et al. (1994) reported the prevalence of p53 gene mutation in major cancers to be ranging from 0% to 60% and some even reached as high as 80% in certain histological subtypes. The high prevalence had contributed to a sizable database whose analysis could yield statistically sound conclusions. In addition, it was highly conserved in vertebrates allowing extrapolation from animal models. Point mutations over a large area of the molecule made it possible to correlate distinct mutations with functional changes (Greenblatt et al., 1994)

B) p53, the discovery

It was difficult not to misclassified p53 as an oncogene with all circumstantial evidences suggesting a transforming role. First, was its detection in animal cells transformed by a virus. The antibody used to immunoprecipitate the Simian virus 40 (SV40) oncogene

product, the large T-antigen, always co-immunoprecipitated a second protein with molecular weight of 53 kDa (hence the name p53) (Lane and Crawford, 1979). McCormik and Harlow (1980) described this to be a host phosphoprotein protein that formed a non-covalent complex with the large T-antigen in the nucleus. Later in the development, the 53kDa protein was again, in the absence of the large T-antigen, detected in murine cells transformed by a chemical agent, methyl-cholanthrene (MethA cells) (DeLeo et al., 1979) and also in several human tumour cell lines (Crawford, 1981). Dippold and colleagues (1981) found high levels of p53 protein in proliferating cells and its perpetual high level in transformed cells as compared to a low level in normal cells after division.

In a series of experiments later, Harlow et al. (1985), Wolf et al. (1985) and Arai et al. (1986) described several complementary DNA (cDNA) and genomic clones of the p53 gene. By using these clones, it was observed that when transfected in cells in culture, p53 was able to immortalize these cells, and with the help of another oncogene, the *ras* oncogene, tumorigenesis occurred (Jenkins et al., 1984; Elihayu et al., 1984; Parada et al., 1984) and hence the conclusion that p53 was oncogenic.

However, p53 finally resumed its status as a tumour suppressor gene when Finlay et al. (1988) and Finlay et al. (1989) demonstrated the existence of the wild-type p53 protein with anti-oncogenic ability coupled by clinical evidences of mutation of the alleles and loss of heterozygosity at the p53 locus in tumour cells (Nigro et al., 1989) which could be restored by returning the wild-type allele to suppress tumorigenic potential of the cells. The gene was subsequently mapped to the short arm of human chromosome 17 at position 17p13.1 with 11 exons producing a protein of 393 amino acids (Levine, 1992)

C) p53 protein

The p53 protein is essentially a transcription factor. The 393 amino acid protein is divided into four structural domains. The four domains with its functions are:

a) N-terminal transactivation domain – essential for association with the basal transcription machinery
b) Central core DNA binding domain - sequence-specific DNA binding domain between amino acids 100-300.
c) Tetramerisation domain – from amino acids 324-355. For tetramerisation of p53 to form dimer of dimers and provides strong binding to the DNA. Also contained nuclear export signal (NES), tetramerisation masks the area and traps p53 in the nucleus.
d) C-terminal – highly basic with 26 amino acids. May influence the conformation of the DNA binding domain via allosteric mechanism, or block the site directly in absence of activating mechanism. (Hickman and Helin, 2000) (Figure 2.3).

D) p53 network

The most important role of p53 in suppressing carcinogenesis was its ability to halt the progression of cell cycle and decide whether to repair the damage or to execute cell death (Wiesmuller, 2001). The p53 protein carries out its functions by either increasing or reducing transcription of its targeted genes in its complex networks of signaling pathways as shown in Figure 2.4. Several complex modifications to the p53 protein by stress signals were necessary to modulate its activity (Giaccia and Kastan, 1998).

In the event of DNA damage, two kinases, the ATM (ataxia-telangiectasia, mutated) and ATR (ATM and Rad3-related), conveyed the message by modifying several amino-acid

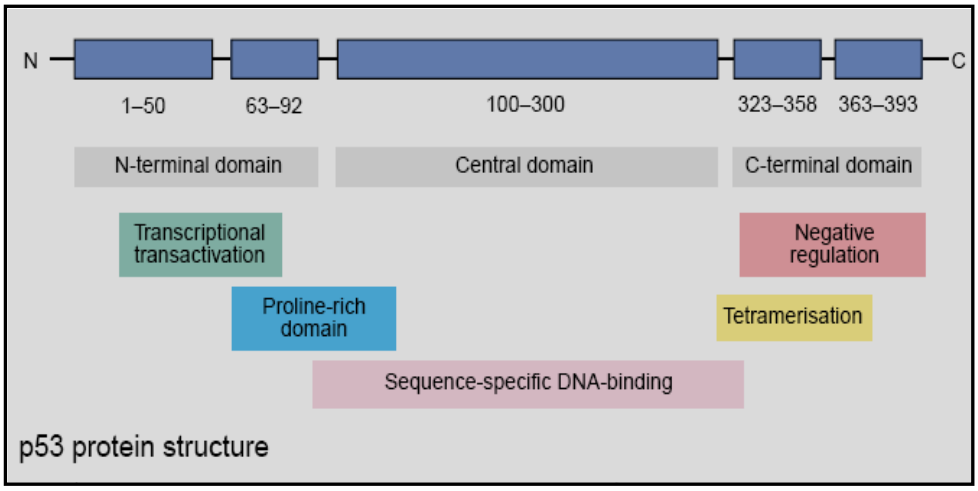

Figure 2.3: The p53 protein structure. Four structural domains depicted. 1) N terminal domain with transcriptional transactivation function 2) Central domain for sequence specific DNA binding 3) C terminal for tetramerisation 4) C terminal for negative regulation on the protein. Adapted from http://www.expertreviews.org.

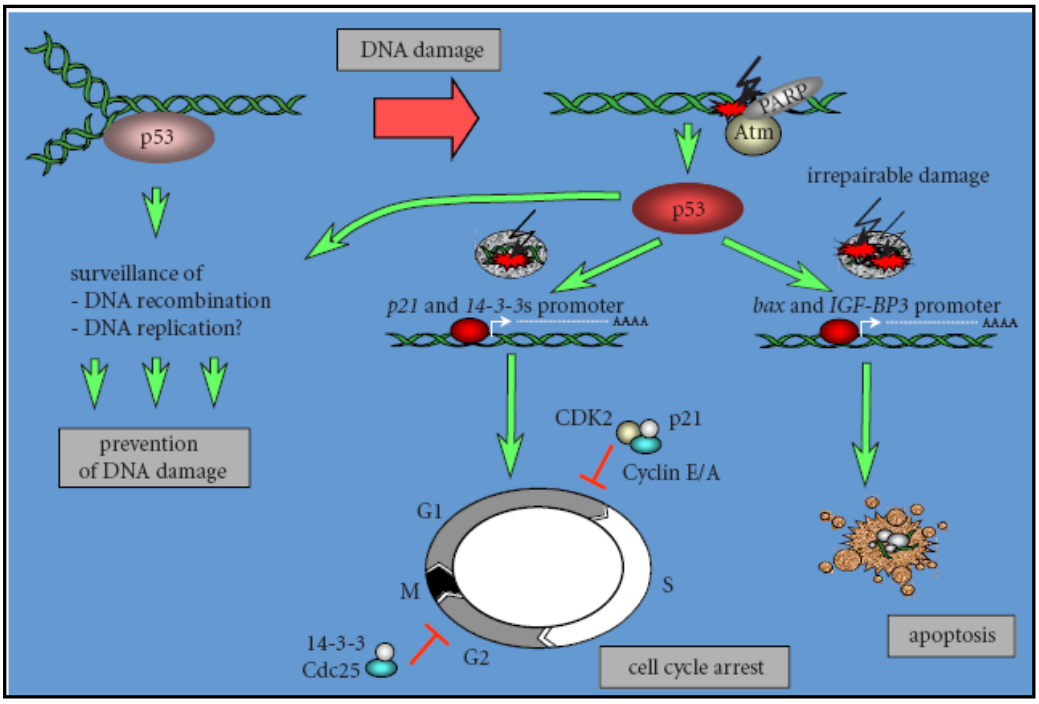

Figure 2.4. Multiple p53 functions in maintaining genomic integrity. Adapted from Wiesmuller, 2001.

of the p53 protein to exert its action on the cell cycle. The ATM and ATR were shown to phosphorylate amino acid Ser15 to enhance its transactivating activity when DNA damage was caused by ionizing radiation and UV radiation respectively. Phosphorylation of amino acid Ser20 was also important in preventing the p53 protein from degradation by its inhibitor, MDM2 (mouse double minute 2) (Shiloh, 2001). The activated p53 protein would in turn increase transcription of the gene p21 and halt the progression of cell cycle in G1/S phase when the p21 protein formed complex with cyclin-dependent kinases and PCNA (Harper, 1997). Koumenis and colleagues (2001) further hypothesized that DNA damages created two pools of p53 protein with one pool capable of interacting with transcriptional corepressors (instead of transactivation) in signaling apoptosis (cell death) dependent of the situation. Subsequent experiment indeed showed that p53 protein underwent modifications allowing it to recognize among different promoters according to the sequence composition of its p53-responsive elements present in the p53-regulated genes (Buzek et al., 2002). Table 2.2 listed a few of the promoters activated and repressed by p53 protein and the list is ever-growing.

E) p53 – a prognostic marker?

It could be appreciated now how mutation in the gene creating a dysfunction p53 protein would bring on genomic instability and tumorigenesis. Missense mutation (79% of mutations) represented the most common mutation in the p53 gene. It was also noted by Greenblatt et al. (1994) that the majority of the mutation was concentrated in several "hotspots" of the most conserved part of the gene leading to the loss of suppressing

Table 2.2: Promoters responsive to p53 protein. Adapted from Hall et al. (1996)

Promoters activated by p53	Promoters repressed by p53 protein
Gadd 45	Bcl-2
p21	DNA polymerase alpha
H-ras	c-fos
MDM2	RB
PCNA	Cyclin A
Bax	c-myc

function of the p53 protein. These hotspots were identified to be corresponded to the region which determined sequence-specific DNA binding and transactivation. Nonmissense mutation was more common in the amino and carboxy termini suggesting that the integrity of a single amino acid residue was not essential to function or conformation.

The detection of p53 protein by immunohistochemistry had been considered the surrogate marker for mutation because it was found that missense mutation often produced a mutant protein with prolonged half life (Hall and Lane, 1994). Many investigators had, since then, assessed the overexpression of p53 protein and its significance in many human cancers.

In breast carcinoma, it was demonstrated that p53 gene was altered throughout disease progression (Davidoff et al., 1991) and its protein overexpression was associated with high grade tumours (Ostrowski et al., 1991; Cattoretti et al., 1988). In addition, Merlo et al.

(1993) suggested mutation in exon 5 and 6 was associated with highly proliferating tumours. Similar overexpression pattern was also noted in colon tumours where p53 was detected in dysplastic epithelial cells in colon polyps suggesting potential malignant transformation in these cells (Van Den Berg et al., 1989; Kaklamanis et al., 1993) and its mutation was found to be associated with shorter survival (Hamelin et al., 1994)

The prevalence of p53 protein overexpression was found to range from 11% to 69% in OSCC (Raybaud-Diogene et al., 1996). It had been reported that this overexpression was seen in dysplastic, potentially malignant oral lesions where some of the tissues underwent malignant transformation (Warnakulasuriya and Johnson, 1992; Regezi et al., 1995). Further studies supported the notion that p53 alteration represented early malignant transformation when it was detected in suprabasal cells in tissues adjacent to the tumour mass (Cruz et al., 2000; Cruz et al., 1998; Shahnavaz et al., 2000). Studies on association of p53 protein overexpression with clinicopathological parameters such as TNM staging, histological grading, and size of tumour, tumour sites, age and sex yielded inconsistent results. The discrepancies were probably due to several factors such as the choice of antibodies used, variations in fixation methods and immunohistochemistry procedures (Raybaud-Diogene et al., 1996; Nylander et al., 2000). However when looking at specific p53 mutations, it was noted that mutations at the core domain (exon 5-8) was associated with shorter survival rate (Yamazaki et a.l, 2003) and more advanced and high grade tumour (Atula et al., 1996). Besides mutation, increased degradation of the p53 protein by MDM-2 protein (a molecule which is involved in the negative feedback loop with the p53 protein) has also been reported for case lacking p53 mutation (Lim et al, 2005).

2.4.2.4 Murine Minute Double 2, the MDM-2 gene

The intricate and delicate relationship between MDM-2 gene and protein with the p53 had been studied extensively. The MDM-2 gene is a downstream effector gene of the p53 protein containing a p53-DNA binding site and a genetically responsive element whose transcription could be activated by recruitment of TRRAP acetyltransferase complexes by the wild type p53 protein (Ard et al., 2002). Its protein, in turn, forms a complex with the p53 protein to reduce p53 protein's ability to act as a positive transcription factor at the MDM-2 gene-responsive element and hence forming an autoregulated feedback loop (Wu et al., 1993).

Overexpression of the gene led to tumorigenesis of many sarcomas (Momand et al., 1998). Physical and functional interaction of the oncoproteins and wild type p53 protein was responsible for its tumorigenicity (Momand et al., 1992). Haines and colleagues (1994) demonstrated that three MDM-2 splice forms, the MDM-2: 1-489, MDM-2: Δ273-298 and MDM-2: Δ314-341, with ability to complex with p53 protein and inhibit its transactivation function.

In order to exert its action on the p53 protein, the MDM-2 protein must gain entry into the nucleus via phosphorylation of its serine 166 and serine 186 by the phosphatidylinositol 3-kinase (PI3-kinase) and its downstream target, the Akt/PKB serine-threonine kinase (Mayo and Donner., 2001). Upon entry, several protein-protein interactions take place between the MDM-2 and p53 protein. Firstly, Chen et al. (1993) showed that amino acid residues 19 to 102 of the MDM-2 protein could bind to amino acid residues 1 to 52 of the N-terminus of the p53 protein. Binding to this transactivation region could be responsible for the inhibition of G1 cell cycle arrest and apoptosis by disrupting the interaction of the p53

protein with the basal transcription machinery (Chen et al., 1996). Secondly, modification at DNA-binding domain (DBD), C-terminus and amino acid residues 219-318 facilitates the export of the p53 protein by MDM-2 or other proteins from nucleus to the cytoplasm (Gu et al., 2001; Freedman and Levine, 1998). Finally, the degradation and abrogation of the functions of the p53 protein by the ubiquitin-proteasome system (Maki et al., 1996) after ubiquitin ligation of p53 C-terminal lysine residues (Rodriguez et al., 2000) which is facilitated by the RING and acidic domain (Honda and Yasuda, 2000; Kawai et al., 2003) of the MDM-2 protein.

A) Expression of MDM-2 protein in cancers

Mapped to human chromosome 12q13-14, a compilation showed that the overall frequency of MDM-2 amplification was 7% in all human cancers with the highest frequency observed in soft tissue tumors (20%), which included Ewing's sarcoma, leiomyosarcomas, lipomas, liposarcomas, malignant fibrous histiocytomas, malignant Schwannomas and other sarcomas such as rhabdomyosarcomas. Osteosarcomas had the second highest frequency of MDM-2 gene amplification (16%). At the other end of the spectrum, several tumors showed no MDM-2 gene amplification, including Wilms' tumors, leukemias, lymphomas, hepatoblastomas and pancreatic carcinomas. Amplification ranged between 2- and 10-fold (Momand et al., 1998).

Ralhan et al. (2000) reported a 7-fold and 9-fold amplification of the MDM-2 gene in hyperplastic oral tissues and OSCC respectively. The group also found an increase in the transcription of the gene resulting in overexpression of the protein detected in hyperplastic, dyplastic and OSCC. Separating young and older patients, Regezi and colleagues (1999) found 11% of the OSCC cases in the less than 35 years group and 28% of the OSCC cases

in the more than 75 year group showed overexpression of the MDM-2 protein. Shibagaki et al. (1995) found that shorter survival rate was associated with esophageal SCC which demonstrated MDM-2 gene amplification; however, Huang et al. (2000) reported no significant association of the overexpression of the MDM-2 protein with any of the clinicopathological parameters in OSCC. Two studies reported shorter survival time in cases demonstrated coexpression of MDM-2 and p53 protein (Ralhan et al., 2000; Agarwal et al., 1999). Recently, Lim et al. (2005) reported a striking 100% overexpression of MDM-2 in OSCC in Malaysia in contrast to a report of 64.5% by Lee (2004) and it was suggested that MDM-2 protein overexpression was the dominant mechanism whereby the suppressive function of p53 protein was inactivated.

2.4.2.5 Apoptosis

Apoptosis, the most predominant form of physiological cell death, is used for the coordinated death of excess, hazardous or damaged somatic cells. The central executors of this process are the caspases (Caspases 2, 3, 6, 7, 8, 9, 10, 12, 13, and 14). These apoptotic caspases undergo activating cleavage during apoptosis and between them they cleave a range of substrate proteins to mediate the apoptotic process. These substrates are grouped according to their functions and two of them are the pro- and anti-apoptotic proteins. Currently, there are two recognized apoptotic pathways:

1. The ancestral pathway. Release of cytochrome c from mitochondria which formed complexed with two cytosolic proteins, the Apaf-1 and -3 which would in turn activate caspase-3 and the apoptotic cascade.
2. The death receptor pathway. This pathway involved the activation of a specific group of transmembrane receptors of the tumour necrosis factor (TNF) receptor

superfamily, either by ligand or by an agonistic antibody (experimentally), leading to direct activation of caspases (Zornig et al., 2001).

2.4.2.6 Bcl-2 protein family

The Bcl-2 family of proteins is central regulators of apoptosis because they integrate diverse survival and death signals that are generated outside and inside the cell. Two classes exist in this family: the anti-apoptotic members such as, Bcl-2 and Bcl-x_L (the Bcl-2-like survival factors) which protects the cells from apoptosis, and, the pro-apoptotic members, the Bax, Bak (the Bax-like death factors) and the large group of BH3-only death proteins which trigger or sensitize for apoptosis (Borner, 2003).

A) Bcl-2

Bcl-2 or the B cell leukemia and lymphoma gene was first discovered in 1984. The translocation of t(14;18) juxtaposes the gene to the immunoglobulin heavy chain and resulting in overexpression of Bcl-2 protein. The altered expression was due to abnormal transcriptional activation and posttranscriptional regulation of Bcl-2 mRNA (Cleary et al., 1986). It was found that Bcl-2 protein expression was needed to expand the life span of epithelial cells with differentiation potential so that proliferation, differentiation and morphogenesis could take place. However, Bcl-2 protein only protected the cells from apoptosis but does not induce proliferation directly. Overexpression of Bcl-2 protein was neither able to immortalize normal epithelial cells nor cause tumorigenic transformation of the immortalized cells (Lu et al., 1996).

The approximately 26 kDa Bcl-2 protein was first found to be an integral-membrane protein and predominantly localized to perinuclear endoplasmic reticulum and a small fraction on the plasma membrane (Chen-Levy et al., 1989). Hockenbery et al. (1990)

reported that Bcl-2 protein actually resided in the inner mitochondria membrane and interfered with apoptosis. However, Krajewski et al. (1993) painstakingly demonstrated the spatial distribution of Bcl-2 protein at outer mitochondria membrane, nuclear envelope and endoplasmic reticulum and it was speculated that Bcl-2 protein was regulating the repartitioning of Ca^{2+} in the event of apoptosis.

B) Bcl-2, mechanism of action

Three mechanisms were proposed for the anti-apoptotic action of Bcl-2 protein (Zornig et al., 2001).

1. Structural similarity between Bcl-x_L and diphtheria toxin and colicin had led to the suggestion that Bcl-2 protein might generate pores in the cytoplasmic and mitochondrial membranes in a voltage- or pH-dependent manner. However, it was Bax (a proapoptotic protein) which was shown to release cytochrome c that led to apoptosis and this release could be blocked by BH-4 domain of Bcl-2 and Bcl-x_L.
2. Based on its analogy with the basal apoptotic machinery of *C. elegans,* Bcl-2/Bcl-x_L might inhibit activation of the apical caspase-9 by the Ced-4 orthologue of Apaf-1. And in this model, the pro-apoptotic members would displace Bcl-2/Bcl-x_L from the Apaf-1/cytochrome c/caspase-9 complex and trigger apoptosis.
3. Bcl-2/Bcl-x_L which resided on the outer surface of the outer mitochondrial membrane might forestall the release of cytochrome c which normally resided in the intermitochondrial membrane space.

(C) Expression of Bcl-2 in OSCC

Like the other candidate genes involved in carcinogenesis, immunoexpression of Bcl-2 had been studied in many cancers. Its detection had been described in 25% of lung SCC (Pezzella et al., 1993), 34% of laryngeal SCC (Hirvikoski et al., 1999) and 58% of esophageal SCC (Obhu et al., 1997). In oral carcinogenesis, Singh et al. (1998) demonstrated its presence in 25%, 32% and 56% of mild, moderate and severe dysplastic tissues respectively. The group also recorded the expression of the protein in 16% of well differentiated, 25% of moderately differentiated and 50% of poorly differentiated OSCC. Jordan et al. (1996) and Yao et al. (1999) supported the observation and reported Bcl-2 protein overexpression in 60% and 50% of OSCC respectively.

While expression of Bcl-2 has an undoubted role in distinguishing reactive lymphoid hyperplasia from follicular lymphoma, its role as a clinical marker in many malignancies is still unclear (Pezzella and Gatter, 1995). In esophageal (Obhu et al., 1997), lung (Pezzella et al., 1993) and laryngeal SCC (Hirvikoski et al., 1999), Bcl-2 protein overexpression was associated with less aggressive tumour, less metastasis to the lymph nodes and longer survival which was in contrast to the association with severely dyplastic tissues, poorly differentiated tumours and more aggressive mode of invasion as in OSCC (Yao et al., 1999; Jordan et al., 1996; Singh et al., 1998). The exact reasons for this discrepancy remain to be elucidated but site specificity and other biological properties might contribute to the differences seen (Hirvikoski et al., 1999; Singh et al., 1998).