

CHAPTER 5: DISCUSSION

Several findings in this study are in concordance with studies previously conducted in Malaysia. These had reinforced several characteristics of OSCC found in Malaysia.

5.1 Sample size

The sample size of this study is relatively small as compared to other studies thus far reported in the literature. In several epidemiological reports, Ng et al. (1985) studied 749 OSCC cases recorded from 1978 to 1984. Ninety five cases from 1978 to 1988 in Siar et al. (1990) report. Khoo et al. (1998) collected 65 cases from 1994 to 1995 for their study regarding diagnostic delay. The current study collected 45 cases selected from year 1998 to 2002. However, these 45 cases were considerably larger when compared to several other more recent studies conducted so far in assessing the roles of molecular markers in OSCC. George (2002) reported on 27 cases in a p53 overexpression study, Abdul Jalil (2003) collected 19 samples from year 1986 to 2002 for a comparison of two proliferative markers in the study and Lee (2004) studied a combination of several markers in 31 cases from year 1986 to 2001.

5.2 Age

This study found no changes in the age of the subjects inflicted by the disease to the question, “squamous cell carcinoma of the oral mucosa in Malaysia – any change?” posed by Ng and colleagues (1985). A majority of the subjects (62.2%) were found in their 5th and

8th decade in the current study. This observation has not changed since 1976 as it is the same as studied by Ramanathan and Lakshimini (1976) whose results were compared in Ng et al. (1985). The current study also recorded six subjects at the age or below 45 years. Even though the cut off age for subjects considered as young was different, the number of cases recorded did not differ much between current study and Siar et al. (1990). It hence can be concluded that OSCC is still a disease of older age group in Malaysia.

5.3 Gender and Ethnicity

There were slightly more male than female subjects in this study with an M: F ratio of 1:0.8. This male preponderance was observed in the West as well (Hindle et al., 1996, Moore et al., 2000). This trend however is different from previous reports. Siar et al. (1990) and Ng et al. (1985) reported preponderance in female subjects. This study recorded an M:F ratio of 1:1.58 in Indian, 1:0.3 in the Malay and all Chinese subjects were male as compared to 1:1.4 for the Indian, 2.0:1 for the Malay and 1:1.1 in Chinese by Ng et al. (1985). The variation could be attributed to the different risk habits as practiced by the different communities. Zain and Ghazali (2001) showed that smoking is highly prevalent in the Malay male and quid chewing in Indian female and alcohol consumption mainly in Chinese and Indian males.

5.4 Sites

Buccal mucosa (42.2%) was the major site for OSCC in this study followed by tongue (24.4%). Interestingly, 73.7% of the buccal mucosa cases were found in the Indian female subjects and 70.0% of tongue cases were found in the Chinese male subjects. This observation was consistent in Malaysia (Ng et al., 1985) and it again reflected the association between risk habit practices and the sites of occurrence of the disease (Lee,

2004) as discussed in 5.3.

5.5 TNM Staging

This study revealed another characteristic trend of OSCC found in Malaysia. George (2002) and Lee (2004) also recorded more late stages cases in their studies. Lee (2004) had 74.2% and George (2002) had 81.5% Stage III and IV cases compared to 68.54% in the current study. The lower incidence in this study was caused by the lack of complete records as some cases were referral for diagnosis from private hospitals and clinics whereby no information were provided for clinical staging. The possible reasons for more late stage presentation were delays in seeking professional help by the patients, and by the clinicians to reach a definitive diagnosis as pointed out by Khoo et al. (1998).

5.6 Broders' Classification & Pattern of invasion

Employing Broders' classification, the current study recorded a high number of cases in the more favourable prognosis category; i.e. the well differentiated (42.2%) and moderately differentiated (55.6%). Only one case belonged to poorly differentiated (2.2%) which carried poor prognosis. Similar observation had been noted in a few recent studies (Lee, 2004; George, 2002). George (2002) recorded 51.9% of well differentiated, 33.3% of moderately differentiated and 14.8% of poorly differentiated cases while Lee (2004) had 67.7% of well differentiated and 32.3% of moderately differentiated cases. Both studies only included OSCC from the buccal mucosa. Comparing to earlier studies, Siar et al. (1990) noted 81.1% of well differentiated, 17.9% moderately differentiated and only 1.0% of poorly differentiated cases taking OSCC of all sites into the study. Broders' classification seemed

to be in favour of a more optimistic prognosis.

However, a different trend emerged when a more recent and better grading system was engaged. Analyzing the pattern of invasion in the current study revealed that a high proportion of the cases (68.9%) contained non cohesive pattern of invasion (55.6% pattern 3 and 13.3% pattern 4). 26.6% of the well differentiated, 40.0% of the moderately differentiated and the only poorly differentiated cases were found to contain cells in small groups and widespread cellular dissociation, i.e. the non cohesive group which is indicative of poor prognosis. This trend was observed in George (2002) as well. Except for Lee (2004) which recorded more cohesive cases (61.3%) than non cohesive (38.7%), George (2002) and the current study recorded 66.6% and 68.9% of non cohesive pattern of invasion respectively. Lee (2004) attributed the difference to the small number of random fields chosen for assessment of pattern of invasion, different time frame from which the specimens were chosen from and difference between incisional biopsy and surgically resected specimens which might influence the scoring of the pattern of invasion. Bryne et al. (1989), however, showed that most, if not all, biopsy specimens with enough underlying connective tissues could be graded. Nonetheless the study emphasized on the importance of surgically removing a large and representative biopsies from the tumours so as to include the metastatic phenotype in a population of heterogenous cells (Anneroth et al., 1987).

5.7 Evaluation of expression of Ki-67, p53, MDM-2 and Bcl-2 at the tumour invasive front and relationship to other clinicopathological parameters

A) Ki-67 Labelling Index

Apart from being perhaps the most commonly used and best known proliferative marker, the significance of Ki-67 immunodetection in prognostic studies was shrouded in uncertainty except for lymphoma and probably breast carcinoma (Hall and Levison, 1990). The other comparable studies to the current one were Lee (2004) and Abdul Jalil (2003) in which materials and methods collected were similar. Ki-67 immunoreactivity was recorded in 62.2% of the cases in this study which falls lower than Lee (2004) (90.3%) and Abdul Jalil (2003) (95.0%). It is also found to be lower than those reported in the literature for example, 100% immunoreactivity in Sittle et al. (1999) and Xie et al. (1999). Brown and Gatter (1990) listed several reasons for discrepancy in detectable Ki-67 immunoreactivity. One of the possible reasons was possible gradual denaturation of the antigen with time even when stored in -70°C . Ki-67 antigen was also known to be sensitive to formalin and would be degraded even in weak form. The best fixation would be by cold acetone for 5-10 minutes as suggested. Nonetheless, Shi et al. (1999) had described a method which had been shown to improve immunohistochemical staining. This microwave oven heating method which is now a common practice, and modified in the current study, in immunohistochemical staining was shown to reverse the deleterious effects of fixation in formalin. Besides antigen decay during storage, cells deprived of nutrition were also found to lose Ki-67 antigen. This is of obvious relevance in cancer study since it is better known that the central areas of solid tumours were usually deprived of nutrients and oxygen (Brown and Gatter, 1990). However, no such information was obtainable for the current study as which part of the tumours the biopsies were taken from. The lower than commonly reported ranged immunoreactivity in this study could be due to site differences as Abdul Jalil (2003) examined only the tongue lesions, George (2002) and Lee, the buccal mucosa while the current study a combination of sites.

A common observation in Abdul Jalil (2003), Lee (2004) and current study was that there was no aberrant pattern of expression of Ki-67 in immunopositive cases whereby brownish staining was recorded only in the nuclei of tumour cells at the tumour invasive front. These studies also found no significant association of the overexpression at the tumour invasive front to any of the clinicopathological parameters investigated. The current study observed a median Ki-67 LI to be 17.8% for well differentiated tumours, 19.4% for moderately differentiated and 22.8% for the poorly differentiated. Since there was only one poorly differentiated tumour in this study, it was not conclusive as to whether the higher Ki-67 LI in the poorly differentiated was of any significance. However, Tumuluri et al. (2002) found that higher Ki-67 LI was significantly associated with poorly differentiated tumours. In this study also a majority of the positive cases (67.9%) were recorded in tumours with invasion pattern 3 and 4 however, the analysis did not reach any significance. The function of Ki-67 in proliferation is still largely unknown. Counting of the number of cells expressing Ki-67 is analogous to a snapshot in time, whereby Ki-67 expression only reveals the number of cells in cycle but does not indicate the time taken for each cells to complete the cell cycle and hence no information is given on the proliferation rate which is associated with the aggressiveness of the tumours (Brown and Gatter, 1990).

B) Expression of p53

Overexpression of the p53 protein was detected in 75.6% of the cases in the current study. This frequency is lower than that reported by George (2002) which had recorded 92.6% of overexpression. It is possible that the difference was attributed to the different genetic expression in different tissues as George (2002) only studied OSCC from the buccal mucosa

but the current study included OSCC from all sites. When analyzing only OSCC from the buccal mucosa, 16/19 cases (84.2%) in the current study overexpressed p53 protein which is close to that of George (2002). However, this frequency is still higher than the reported range of most studies in the literature, 11-69% in OSCC as reviewed by Raybaud-Diogene et al (1996). Baas et al. (1994) explained that the discrepancy was caused by, first of all, the use of different primary antibodies since each antibody recognized different epitopes in the p53 protein, in addition p53 binding to other proteins and macromolecules would limit accessibilities of specific antibodies to the target epitopes; secondly, no doubt the use of antigen retrieval agent enhanced immunostaining and reduced background staining, the efficiency, however, was shown to varied in different antibodies and DO-7 was shown to benefit the most from the method. Dowell and Ogden (1996) demonstrated further that antigen retrieval method indeed increased immunoreactivity of p53 but in both benign and malignant tissues. The detection of immunoreactivity in benign tissues could be due to the lowering of the detection threshold and a reflection of normal cellular function of the wild-type p53 protein rather than gross tumour-associated overexpression. Fisher et al. (1994) added that certain fixatives like phenol formol saline was the best in maintaining cell morphology and preserving antigenicity for a wide range of antibodies including p53. The group also demonstrated that optimally fixed tissues gave crisp clear nuclear staining as compared to the poorly fixed with occasional leakage of the protein from nucleus to the cytoplasm resulting in cytoplasmic staining. Tissues fixed in neutral buffered formalin suffered from background staining problem. Other problems affecting the results included were power of the source, temperature, section/buffer ratio and also the “adjusting” of primary antibody dilution to achieve the “appropriate” results hence comparing results among studies became difficult when there was no rigidly standardized protocol to be

adhered to (Allison and Best, 1998). Baas et al. (1994) also demonstrated that mutation producing a stop codon thus truncating the protein and making accumulation and overexpression impossible or a nonmissense mutation resulting in a mutated protein that did not contain nuclear localization domain and gross deletion which abolished p53 protein production, could all result in complete lack of immunoreactivity.

Immunohistological assessment of the p53 protein had become popular as a surrogate marker for missense mutation in the gene which caused stabilization and accumulation of the protein (Baas et al., 1994). The popularity was driven by the relative simplicity of the methodology and rapidity of the results (Hall and Levison, 1990) and also the availability of the reagents (Save et al., 1998). Unfortunately, Hall and Lane (1994) raised concern about equating p53 protein stabilization and detection by immunohistochemistry with mutation. In the editorial, gathering evidences, they concluded that besides mutation which abolished its function and leading to its stabilization, cellular environment was another determinant of p53 stability. Indeed, in a report by Lim et al. (2005), only 30% of the cases had mutation in the p53 gene. In summary, critical protein-protein interaction with the MDM-2 protein, HPV viral protein and genotoxic damage could also result in an increase and stabilized p53 protein (Hall and Lane, 1994). Adding to the complexity was the discovery of p53 homologues like p73 alpha, p73 beta and KET which bore many sequence and probably structural features in common with p53 (Save et al., 1998).

The subcellular distribution of p53 immunoreactivity in this study had been exclusively in the nuclei and confined to the tumour invasive margin as observed in George (2002). No cytoplasmic staining was observed. It was also noted that immunoreactivity in large tumour

islands was diminishing towards the central, more differentiated part of the tumours (George, 2002). This observation is in agreement with the protein's function as a transcription factor (Hall et al., 1996).

Like many other studies, there was no observed relationship between the overexpression of p53 protein with any clinicopathological parameters (Raybaud-Diogene et al., 1996). However, the overexpression was seen in 71.4% of non cohesive tumours. Zariwala et al. (1994) and George (2002) had also demonstrated this trend. It seems, thus far, that Ki-67 and p53 activities may influence the morphology at the tumour invasive front, a notion put forth by Bryne (1998).

C) Expression of MDM-2

The finding in overexpression of MDM-2 is perhaps the most striking in the current study. Forty four cases (97.8%) were shown to overexpress the protein. Except for Lim et al. (2005) which recorded 100%, other studies recorded 64.5% (Lee, 2004), 78% (Agarwal et al., 1999), 71% (Ralhan et al., 2000) and 69% (Huang et al., 2001) immunoreactivity. There could be technical and biological explanations for the observed difference in immunoreactivity. First of all, the use of different antibodies. Lim et al. (2005) and current study had used mouse monoclonal anti-MDM-2 (code 1B10) antibody as the primary antibody while Lee (2004), Agarwal et al. (1999), Ralhan et al. (2000) had used SMP14 monoclonal antibody. These two antibodies recognized different epitopes in the MDM-2 protein. Anti-MDM-2 antibody 1B10 detected epitopes at the C-terminal of the protein and SMP14 recognized epitope 154-167 amino acids (Agarwal et al., 1999). Gorgoulis et al. (1996) had used 1B10 antibody and IF2 (similar to SMP14 which detects N-terminal of the

MDM-2 protein) in order to avoid false-negative results due to production of MDM-2 proteins with truncated N- or C-terminal and found 66% cases overexpressing the protein. Secondly, the use of different detection system. DakoCytomation EnVision[®] and System-HRP as used in current study and Lim et al. (2005) is an extremely sensitive method compared to the traditional Avidin-Biotinylated Complex (ABC) method as employed by the other studies (Lee, 2004; Agarwal et al., 1999; Ralhan et al., 2000). And the importance of careful definition of the sensitivity of the detection system had been highlighted by Hall and Lane (1994). Ralhan et al. (2000) noted the different subcellular localization of the MDM-2 protein isoforms. Isoform p90 (the full length of the MDM-2 protein) and p57 (devoid of the C-terminal) was found to be present in the nucleus or nucleus and cytoplasm, whereas isoform p76 (devoid of the N-terminal) was found in the cytoplasm and plasma membrane. Deducing from this observation, the current study could have detected the p90 isoform of the MDM-2 protein since the immunoreactivity was confined exclusively to the nuclei and since 1B10 could not detect isoform p57 which lacked of the C-terminal that the antibody recognized.

Another interesting finding, besides the complete immunoreactivity which was confined exclusively to the nuclei, was the distribution of the immunoreactivity. Every nucleus in every positive case showed brownish staining. Lim et al. (2005) shared the same observation except for in the report; the group also noted occasional nuclear and cytoplasmic staining. Gorgoulis et al. (1996) also noted overexpression of the MDM-2 protein in normal bronchial epithelium besides neoplastic areas. Ralhan et al. (2000) reported no notable MDM-2 overexpression in normal oral tissues but noted faint overexpression in the normal epithelium adjacent to the dysplastic lesions. Dazard et al. (1997) elegantly demonstrated

that MDM-2 was present in the keratinocytes in the different layers of epithelium and p90 was the major form detected. Hence, this could be the reason for the detected immunoreactivity in the suprabasal layers of the tumour epithelium as observed in this study or it could also be a reflection of an appropriate response to external and internal stimuli by the epithelium (Price et al., 1994).

D) Expression of Bcl-2

The very low expression as observed in the current study is indeed unprecedented in the literature. Only 2 cases (4.4%) showed immunoreactivity in this study as compared to 16.1% in Lee (2004). Lo Muzio et al. (2003) reported 17% of immunoreactivity from 90 OSCC cases while the highest reported immunoreactivity (100%) was by Nakagawa et al. (1994). Popovic et al. (2005) also reported low percentage of positive cases with mean value ranging from 7.5 to 34.4 from an assorted OSCC in different stages. It is difficult to make comparison with other studies for example; Nagakawa et al. (1994) assessed only 4 cases of OSCC of which two were frozen tissues and the other were squamous cell carcinoma cultured cell line. The only comparable study will be Lee (2004) whereby the protocol used was the same. The most likely explanation for the discrepancy in immunoreactivity would be the samples in Lee (2004) study comprised mainly of surgically resected tumours while the current study included only biopsied tissues. It was possible that due to the heterogenous nature of tumours the biopsies did not include the phenotypic variants which overexpressed Bcl-2 (Bryne et al., 1989) Another possible reason for discrepancy was the presence of different isoforms of Bcl-2 protein as a result of alternate mRNA splicing and possible elective capase activation resulting in proteolytic degradation of bcl-2 in tumour cells as highlighted by Loro et al. (1999). These isoforms and cleaved variants could affect the

sensitivity and specificity of the available Bcl-2 antibodies.

It is indeed intriguing to see the vast difference in the two reports whereby patients' selection, etiological factors and laboratory protocol were all similar. What is more intriguing is the exclusive nuclear staining as recorded by Lee (2004). This study observed the exclusive staining in the cytoplasm. Lee (2004) was not the only study which noted nuclear staining (Chan et al., 1995). Kannan et al. (1998) reported the same observation in the OSCC in the Indian patients. This "unusual" pattern of staining had resulted in Saranath (1999) questioning the validity of the antibody and the probable non specific binding of the antibody to nuclear protein in Kannan et al. (1998) study. Shanmugam (1999) from the group quickly refuted that nuclear staining noted in the report would not be a result of non specific reaction with nuclear protein as some of the cases were completely devoid of staining. What was not highlighted was the elegant demonstration of Bcl-2 localization to the chromosomes of dividing epithelial cell lines by Lu et al. (1994). The group had detected the presence of Bcl-2 in the chromosome of mitotic nuclei and especially strongest from prophase to metaphase. Although not observed in the tissues hitherto, Lu et al. (1994) hypothesized a protective role of the presence of Bcl-2 in the mitotic cells from programmed cell death and hence might play a role in cell survival and immortalization in vitro.

Since there were only two positive cases in this study, it was not very informative when the relationship with Broder's classification and pattern of invasion was investigated. The two positive cases were both graded as moderately differentiated tumours with pattern of invasion type 1 and 3. The overexpression was distributed along basal and suprabasal layers at the tumour invasive front. One of the tumours exhibiting pattern of invasion type 1 had

higher Ki-67 LI (64.6%) than the one with type 3 pattern of invasion (19.4%). Yao et al. (1999) had observed the same pattern of distribution in all layers of dysplastic epithelial adjacent to the tumours in tongue. And the group also found association between the overexpression and poorly differentiated and more invasive tumours. Other reports investigated the involvement of Bcl-2 expression in the progression of oral carcinogenesis. Singh et al. (1998) reported the proportionate increase in Bcl-2 immunoreactivity from basal to suprabasal layers as the lesions progressed from mild to severe dysplasia and also observed more poorly differentiated carcinoma exhibiting the protein. However, the overall overexpression of Bcl-2 was lower in carcinomas than dysplastic tissues. In another report, Birchall et al. (1997) observed the same reduced expression of Bcl-2 in the carcinoma and also in the related “normal” epithelium adjacent to the tumours and suggested the need to investigate the level of other players such as Bax and p53 in the apoptotic pathway. The presence of Bcl-2 in abundance in the basal and suprabasal layers in the dysplastic tissues could indicate its role in conferring growth advantages in these cells for further clonal expansion when additional malignant changes occurred (Singh et al., 1998).

5.8 Evaluation of coexpression Ki-67, MDM-2, p53 and Bcl-2 at the tumour invasive front in relation to clinicopathological parameters.

A) MDM-2 and p53 expression

Patterson and colleagues in 1996 suggested that aetiological and ethnic differences might play a role in the molecular changes brought about in oral cancer. In that report, they highlighted the low p53 mutation incidence in the India subcontinents as compared to the West. There are indeed evidences now that besides mutation, other mechanisms (Bolt et al.,

2005; Hoque et al., 2002) are involved in abolishing the tumour suppressive function of the p53 protein and one of the mechanisms is overexpression of the MDM-2 protein. The presence of MDM-2 protein in excess could be responsible for the observed overexpression of the p53 protein in 35 cases of this study by binding and stabilizing the wild type p53 leading to its accumulation (Ralhan et al., 2000). Current study supported Lee (2004), Lim et al. (2005), Ralhan et al (2000) and Agarwal et al (1999) in that, at least in a subset of OSCC cases, overexpression of MDM-2 protein is the mechanism disrupting the p53 pathway. The striking resemblance among the studies is the ethnicity and risk habits the subjects were exposed to. Although not recorded in the present study, other studies had reported the overexpression of MDM-2 protein in tobacco and quid related OSCC (Agarwal et al., 1999; Lim et al., 2005, Ralhan et al., 2000; Pande et al., 2002; Huang et al., 2000). MDM-2 amplification leading to its overexpression was found to be infrequent in OSCC (Ralhan et al., 2000), hence it could be speculated that the abundant MDM-2 protein in this study was caused by activation of the second p53-responsive promoter in the MDM-2 gene resulting in an enhanced translation of the transcripts. Mutational analysis on the p53 gene status was carried out in the current study however, unfortunately, DNA amplification from microdissected cells was unsuccessful and hence no information was available regarding the exact mechanism behind p53 protein stabilization. Although there was no significant correlation between the overexpression of these two proteins to any of the clinicopathological parameters as in Huang et al. (2001); Agarwal et al. (1999) and Ralhan et al. (2000) found that they correlated to more advanced tumour, lymph node metastasis and also shortened survival time respectively. With this, the dysfunction p53 protein might be responsible for several other observations in this study.

B) p53 expression, Ki-67 LI and Bcl-2 expression

The lack of p53 protein function would carry detrimental results to the cellular system under carcinogenic insult. As depicted in Figure 2.4, p53 is the main executor in directing cellular responses to various stresses encountered. Two very important responses would be cell cycle arrest and apoptosis. The p53 protein carried out these responsibilities by inducing its downstream target genes as outlined in Table 2.2. In the normal circumstances, with functioning wild type p53 protein, cells under oncogenic stresses would be expected to stop cycling under the influence of the p21 protein. In the present study, 21 out of the total cases co-expressed Ki-67 and p53 protein (46.7%). The median Ki-67 LI was 17.6% in cases overexpressing p53 and 22.05% in cases with negative immunostaining of the p53 protein. This encounter could be attributed to the fact that p21 protein was not induced in the absence of functioning p53 protein caused by an overexpression of the MDM-2 protein (in positive p53 cases) or a missense mutation resulting in truncated p53 protein (p53 negative cases). Hence, in both scenarios there was a continuous proliferation as indicated by the presence of Ki-67. Though no relationship was found between the coexpression of Ki-67 and p53 to any of the clinicopathological parameters, it was also observed that a majority (14 out of 31 cases) of the coexpressed cases were found in non cohesive pattern of invasion.

Investigating further the effect of the dysfunction p53 protein revealed that, in Bcl-2 overexpressed cases, one demonstrated p53 accumulation and the reverse in another. The coexpressed case was found in tumour with pattern of invasion type 3 with a Ki-67 LI of 19.4% while the one without p53 overexpression was found in tumour with pattern of invasion type 1 and a Ki-67 LI of 64.6%. The detected p53 in the coexpressed subject might represent the stabilization of the wildtype p53 protein under oncogenic stress and hence

retained its tumour suppressive function causing a lower Ki-67 LI, while the one without the presence of overexpression of the p53 protein coupled with overexpression of Bcl-2 had escaped both tumour suppression and apoptosis and resulting in a higher Ki-67 LI. As the accumulation of the Bcl-2 protein in both cases were in the basal and suprabasal layers at the tumour invasive front, it could indicate that the overexpression might represent an early event in oral carcinogenesis to confer growth advantages for the progression and emergence of a more invasive phenotype (Singh et al., 1998). Another area to be investigated would be the expression of Bax, a downstream target of the p53 protein which is proapoptosis. Bax acts on Bcl-2 to counter the antiapoptotic effects of Bcl-2 and studies had shown that the ratio of Bax/Bcl-2 might play a role in OSCC (Loro et al., 1999).