

CHAPTER 3: MATERIALS AND METHODS

This study has been approved by the medical ethics committee, Faculty of Dentistry, University of Malaya. (MEC Approval Number: DFOP0301/0001(P)).

3.1 Samples selection

Forty five biopsy oral squamous cell carcinoma samples collected were formalin fixed; paraffin embedded archival tissues from the Diagnostics Laboratory, Faculty of Dentistry, University of Malaya. Samples were collected following the criterias:

A) Inclusion criteria

- a) Paraffin embedded specimens from oral cancer patients who have not received preoperative radiotherapy, chemotherapy and surgery other than routine dentoalveolar procedures and a recent diagnostic biopsy.
- b) The sections from these specimens showed enough underlying connective tissues so as to include the most invasive front for evaluation of pattern of invasion.

B) Exclusion criteria

- a) Specimens from oral cancer patients who have undergone preoperative radiotherapy, chemotherapy and surgery.
- b) Specimens that did not show enough underlying connective tissues for evaluation of pattern of invasion.

3.2 Data collection

Data collection from patients' biopsy reports and folders had been approved by the Dean of Faculty of Dentistry, University of Malaya (2003). These data included:

- 1) Sociodemographic – age, sex, gender, ethnic groups and site of lesions
- 2) Histopathological grading – Broders classification and pattern of invasion.

Definition of sites of lesions will follow guidelines provided by International Classification of Disease for Oncology (ICD-10). (See Appendix 3 for site specification)

Histopathological grading from biopsy reports of the patients were reassessed by the author and an oral histopathologist (RMZ) after a few sessions of training and calibration using criteria as follows:

- 1) Broders' classification – according to Pindborg et al (1997), grading of the malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges by subjectively assessing the degree of keratinisation, cellular and nuclear polymorphism and mitotic activity. Tumours were graded as:
 - a) Grade I: Well differentiated – histological and cytological features closely resemble those of the squamous epithelial lining of the oral mucosa. There are varying proportions of basal and squamous cells with intercellular bridges; keratinisation is a prominent feature; few mitotic figures are seen and atypical mitoses or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal
 - b) Grade II: Moderately differentiated – this is a neoplasm with features intermediate between well differentiated and poorly differentiated. Compared with well-

differentiated squamous cell carcinoma, these have less keratinisation and more nuclear and cellular pleomorphism; there are more mitotic figures and some are abnormal in form; intercellular bridges are less conspicuous.

c) Grade III: Poorly differentiated – histologically and cytologically there is only a slight resemblance to the normal stratified squamous epithelium of the oral mucosa. Keratinisation is rarely present and intercellular bridges are extremely scarce; mitotic activity is frequent and atypical mitoses can readily be found; cellular and nuclear peomorphism are obvious and multinucleated cells may be frequent. (Figure 3.1)

2) Pattern of invasion – assessing the cohesiveness of the tumour (Crissman et al, 1984). Modified by Anneroth et al (1987) and introduced by Bryne (1998) as part of the Invasive Front Grading (IFG) system. Four different recognizable patterns are:

a) Pattern I – pushing, well delineated infiltrating borders

b) Pattern II – infiltrating, solid cords, bands and/or strands

c) Pattern III – small groups or cord of infiltrating cells ($n < 15$)

d) Pattern IV – marked and widespread cellular dissociation in small groups and/or in single cells ($n < 15$). (Bryne, 1998). (Figure 3.2)

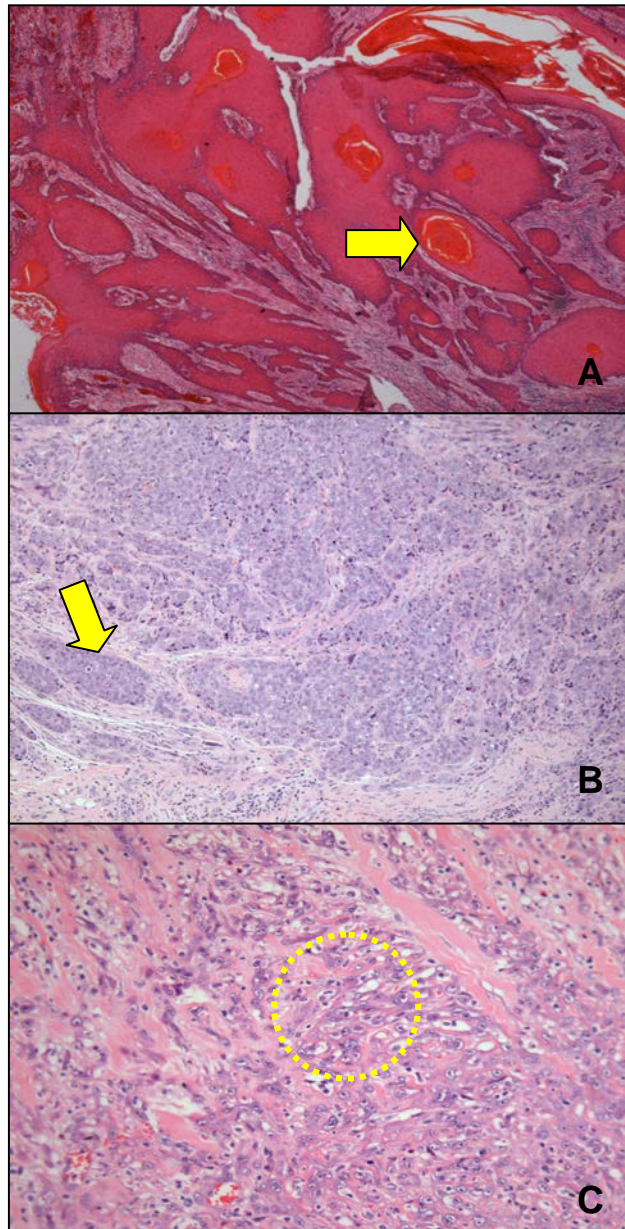


Figure 3.1: Broders' Classification of OSCC. A. Well differentiated OSCC, tumour islands showing prominent keratinisation (arrow) with minimal nuclear and cellular pleomorphism. B. Moderately differentiated OSCC, tumour islands showing less keratinisation with more nuclear and cellular pleomorphism (arrow). C. Poorly differentiated OSCC, histologically and cytologically there is only a slight resemblance to the normal stratified squamous epithelium of the oral mucosa, tumour cells showing less cohesiveness of cells (circle). (Original magnification, 64x)

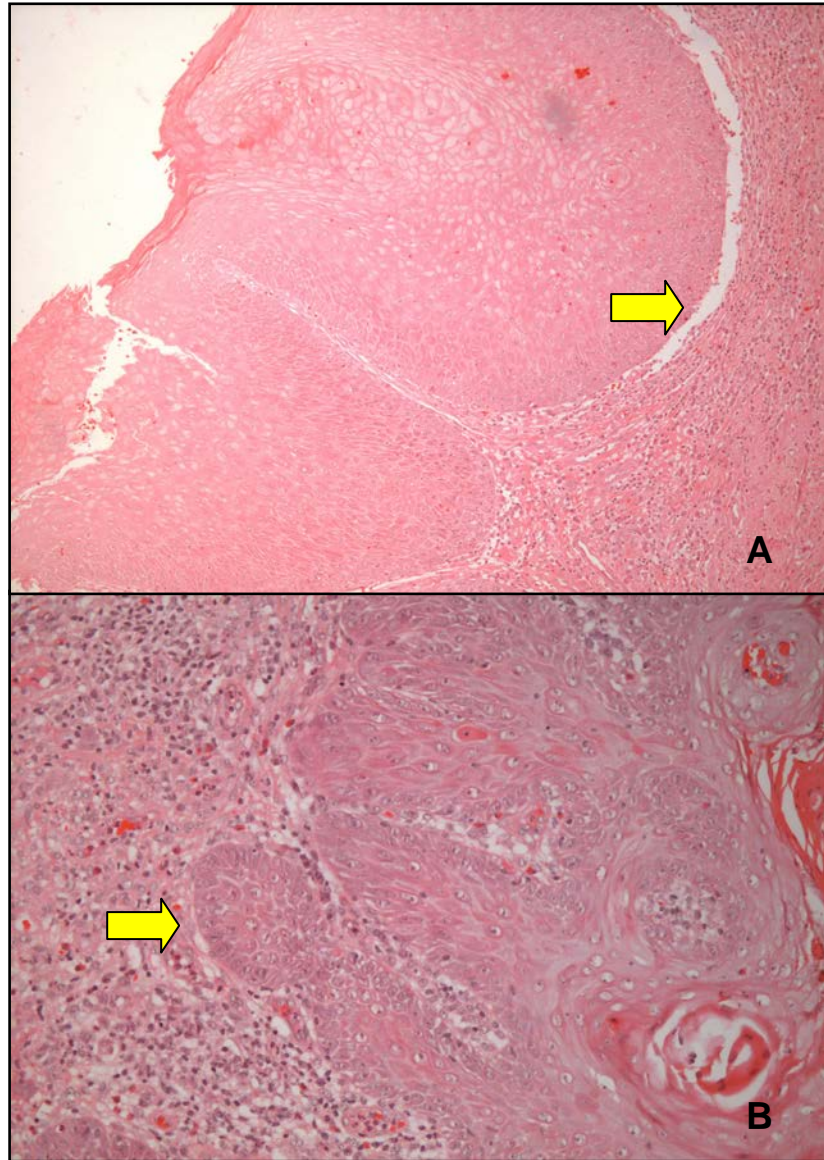


Figure 3.2 Pattern of invasion. A. OSCC with well delineated and pressing borders (Pattern 1, cohesive). B. OSCC invading in solid cords and strands (Pattern 2, cohesive). (Original magnification 90x) (Arrow showing pattern of invasion).

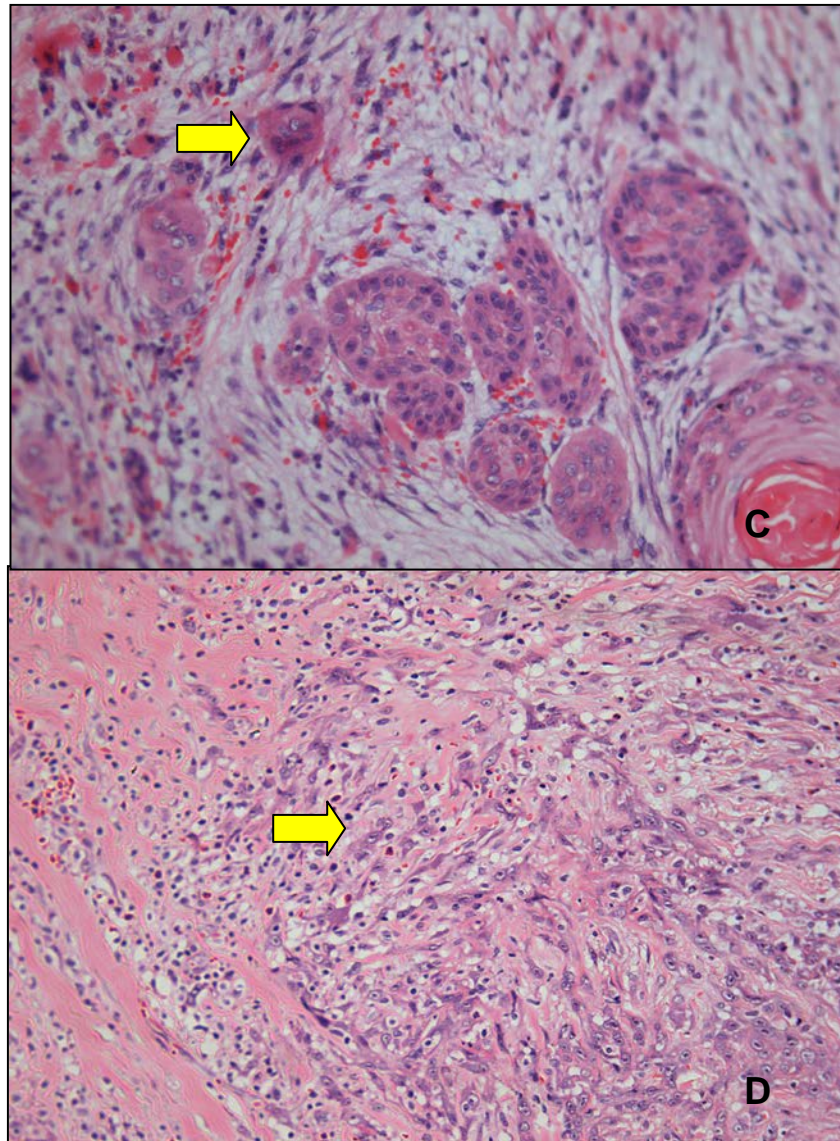


Figure 3.2. C. OSCC in small groups of cells or cords ($n < 15$) (Pattern 3, non cohesive). D. OSCC invading in small groups or single cell ($n < 15$) (Pattern 4, non cohesive). (Original magnification 90x) (Arrow showing pattern of invasion).

3.3 Laboratory procedures

All the samples were subjected to immunohistochemical staining to detect the expression of Ki-67, p53, MDM-2 and Bcl-2 separately. A uniform scoring system was used to evaluate cells showing positive nuclear and cytoplasmic staining

3.3.1 Specimens processing

All archival samples from the Diagnostics Laboratory had been routinely fixed in 10% buffered formalin and embedded in paraffin wax blocks. Sections of 4µm thickness were cut from the blocks and mounted on sialinized slides. (See sialinized slides processing in Appendix 1).

3.3.2 Staining procedures

a) Haematoxylin and Eosin (H&E) staining

Several samples underwent H&E staining for evaluation of the histopathological parameters as some previous slides were contaminated while kept in the Laboratory's library. See H&E staining method in Appendix 2.

b) Immunohistochemistry staining

i) Ki-67

Sections were prepared and stored in 37°C oven overnight. They were then heated up in 60°C oven for 3 minutes before dewaxing in 2 xylene baths for 5 and 4 minutes each. Rehydration followed in decreasing grades of ethanol; 100% for 3 minutes; 95% for 3 minutes and finally 75% for 3 minutes. Sections were brought into running tap water for 3 minutes before being immersed in 10mM citrate buffer at pH6.0 for heating in a

microwave oven for antigen retrieval. Antigen retrieval was carried out for 20 minutes at temperature between 95°C to 100°C.

After antigen retrieval, sections still immersed in citrate buffer was let to cool down to room temperature in about 45 to 60 minutes. They were then washed in running tap water and reimmersed in 3% hydrogen peroxidase in methanol for 20 minutes to block endogenous peroxidase activity. After that, the sections were washed twice in Tris Buffered Saline (TBS) pH7.6 1 minute each. Incubation with normal swine serum at dilution 1:20 followed to block non-specific binding and to reduce background staining for 20 minutes in a humidified chamber.

Sections were drained from excessive serum after 20 minutes and followed by incubation with the primary antibody; the polyclonal rabbit anti-human Ki-67 antigen from DAKO clone MIB-1 diluted in 1:100 for 45 minutes. The use of a humidified chamber was to ensure that slides would not dry out during the incubation time. After the incubation, all sections were washed again twice in TBS for 1 minute each and subsequently incubated with the secondary antibody; the biotinylated swine anti-rabbit from DAKO diluted in 1:300 for 30 minutes.

Meanwhile, Avidin Biotin Complex/Horseradish Peroxidase from DAKO was prepared 30 minutes before use according to the manufacturer's instruction. It was then applied to the sections for 30 minutes after the sections were cleaned from the secondary antibody in TBS twice for 1 minute each.

Finally, the peroxidase activity were developed in 3, 3'-diaminobenzidine (DAB) (diluted 1:50) for 10 minutes after TBS washing. Sections were cleaned from DAB in running tap water and brought to counterstain in Herlich's haematoxylin for 1 minute, followed by 10 dips in 0.5% acid alcohol to remove superficial haematoxylin. They were then rinsed in running tap water for 3 minutes before dipping into 2% sodium acetate to enhance nuclear staining. Sections were then dehydrated in increasing grades of ethanol and immersed in 3 changes of xylene for 2 minutes each before mounted using DPX.

An estimated of 200µl of antibody was needed to fully cover the tissues for maximum reaction and the volume varied according to the size of the tissues and all reagents were diluted using TBS. Normal tissues from impacted wisdom teeth removal or collected under operculectomy were included. Positive procedural control was taken from oral squamous cell carcinoma tissue known positive for Ki-67 antibody from previous study (Lee, 2004). Primary antibody was omitted and replaced by TBS during incubation for negative procedural control.

ii) p53

Similar procedures were carried out for p53 immunostaining. Several changes were made:

- 1) normal rabbit serum instead of normal swine serum was used to block background staining
- 2) primary monoclonal mouse anti-human p53 antibody from DAKO clone DO-7, diluted at 1:100 incubated for 2 hours
- 3) biotinylated rabbit anti mouse replaced swine anti rabbit for secondary antibody incubation

- 4) positive control was OSCC tissue known positive for p53 from a previous study (George, 2002).

iii) Bcl-2

All procedural steps remained the same for detection of Bcl-2 expression except in:

- 1) primary monoclonal mouse anti-human Bcl-2 antibody from DAKO clone 124 diluted at 1:100 incubated for 45 minutes
- 2) detection of primary antibody by EnVision[®] Detection System, Peroxidase/DAB, Rabbit/Mouse, ready-to-use incubated for 30 minutes.
- 3) Positive control was lymph node tissue known to be positive for Bcl-2.

iv) MDM-2

Same procedures as described above were applied to detect expression of MDM-2 except for:

- 1) primary monoclonal mouse anti human MDM-2 antibody from Novocastra clone 1B10 diluted at 1:150 incubated for 30 minutes
- 2) use of EnVision[®] Detection System, Peroxidase/DAB, Rabbit/Mouse, ready-to-use incubated for 30 minutes to detect primary antibody
- 3) positive control was OSCC tissue known positive for MDM-2 from previous study (Lee, 2004).

3.5 Histopathological and Immunohistochemistry Evaluation

A) Broders' Classification

Three randomly chosen fields were viewed under high power (400x) to evaluate the grade of the tumours i.e. Grade 1 – well differentiated, Grade 2 – moderately differentiated and Grade 3 – poorly differentiated. The worst tumour grade of the 3 fields was taken as the final tumour grade.

B) Pattern of invasion

Three randomly chosen fields at the tumour invasive front were viewed under magnification 100x to evaluate the pattern of invasion according to the criteria described previously. The worst pattern was taken as the final pattern of the tumour.

Evaluation was done by two independent observers, the author and an oral pathologist (RMZ) without reference to the biopsy reports after a series of training and calibration. Results were compared and disagreements were discussed to reach consensus.

C) Immunohistochemistry- Evaluation

Evaluation of immunoreactivity of Ki-67, p53, MDM-2 and Bcl-2 were determined at the advancing front of the tumours. Three randomly selected fields were viewed under magnification 400x with an aid of an image analyzer. Cells were considered as positive for Ki-67, p53 and MDM-2 if dark brown nuclear staining was noted and positive for Bcl-2 if brown cytoplasmic staining was seen. Any deviations from these observations were taken note of as well.

Guidelines for scoring immunohistochemical staining was modified from van Diest et al (1997). A clear and sharp image of the field was captured and its maximum border outlined at the advancing tumour front. Positive nuclei/cytoplasm and total numbers of cells on 3-6 layers of cells at the tumour advancing front, were counted in the field. The percentage of positive nuclei/cytoplasm was counted using the formula as follows:

$$N \text{ (number of positive nuclei/cytoplasm)} / M \text{ (total number of cells)} \times 100\% = \% \text{ of positive nuclei in first field.}$$

The percentage of positive nuclei/cytoplasm in two other fields was calculated in the same manner. And the final percentage for all samples would be the average percentage taken from the three fields. Subsequently a scale of 1+, 2+ and 3+ was given to each sample according to the final percentage as showed in Table 3.1. Cases were taken as positive if > 10% of the cells shows immunoreactivity.

Table 3.1: Uniform scoring system for positive nuclei/cytoplasm of p53, MDM-2 and Bcl-2. (Adapted from Ng et al, 1999)

Scale	% of positive nuclei
1+	< 20%
2+	20-50%
3+	> 50%

Ki-67 Labeling Index (LI) is the ratio of Ki-67 positive nuclei to the total number of the cells in the chosen field and is expressed in percentage. The average percentage of 3 fields was taken as the final percentage and LI for the sample.

3.6 Statistical analysis

Pearson's Chi-square, Fisher's exact and Mann-Whitney U tests were employed for statistical analysis of categorical and continuous data. Significance was calculated and p value of <0.05 were considered to be significant.