CHAPTER THREE

MATERIALS and METHODS

This is a preliminary study which is part of a major project on "Oral Cancer and Precancer In Malaysia- Risk Factors, Prognostic Markers, Genetic Expression and Impact On Quality of Life". MEC ethics approval number is **DF OS0905/0017(p) at 15th of May 2009.**

Additional ethnics approval for this part of project was obtained from the ethics committee, Faculty of Dentistry, University Of Malaya.

3.1. Materials:

This study, is based on 50 OSCC paraffin embedded tissue samples of surgical specimens (resected tumors) available in the archives of the Oral Pathology and Oral Medicine department and Oral Cancer Research Coordinating Center (OCRCC) from the year 2004 to 2010. All the tissues were previously fixed in 10% buffered formalin and processed with standard histological embedding techniques. Power and sample size calculation software V2.1.31 was used to obtain a minimal size sample of 45. However, we round up the number to 50 in order to have equal number of samples for tongue and B.M. SCC.

3.1.1. Inclusion criteria:

- Archival tissue from primary squamous cell carcinoma (OSCC) of the tongue and buccal mucosa taken in this study which were never exposed to radiotherapy or chemotherapy treatment previously.
- Cases of histopathologically confirmed SCC of tongue and buccal mucosa from 1st January 2004 to 1st June 2010.

3.1.2. Exclusion criteria:

- Samples from patients who have been exposed to radio/chemotherapy.
- Samples from recurrent oral squamous cell carcinoma despite the sites mentioned previously.

3.1.3. Clinicopathological data:

Data on demography, tumor site, histopathological grade, pattern of invasion, tumor depth, pathological TNM staging and survival were obtained from the Malaysian Oral Cancer Tumor and Database (MOCTDBS) and reevaluation of the histopathological data were done by the author and the second supervisor (an experienced pathologist) to increase the reliability in the present study.

3.1.3.1. Modified Broder's malignancy grading system:

This grading system was used to evaluate the histopathogical grading. It subjectively assesses the degree of differentiation and keratinization of tumor cells (Pindborg & Reichart 1997). There are three grades in this grading system:

Grade 1: Well-differentiated: Histological and cytological features closely resemble those of squamous epithelial lining of the oral mucosa. There are varying proportions of basal and squamous cells with intercellular bridges; keratinization is a prominent feature; few mitotic figures are seen and atypical mitosis or multinucleated epithelial cells are extremely rare; nuclear and cellular pleomorphism is minimal.

Grade 2: Moderately differentiated: This is a neoplasm with features intermediate between well and poorly differentiated. Compared with well-differentiated squamous cell carcinomas, these have less keratinization and more nuclear and cellular pleomorphism; there are more mitotic figures and some are abnormal in form; intercellular bridges are less conspicuous.

Grade 3: Poorly differentiated: Histologically and cytological there is only a slight resemblance to the normal stratified squamous epithelium of the oral mucosa. Keratinization is rarely present and intercellular bridges are extremely scarce; mitotic activity is frequent and atypical mitoses can readily be found; cellular and nuclear pleomorphism are obvious and multinucleated cells may be frequent.

3.1.3.2. Pattern of invasion:

According to the Royal College of Pathologists (Helliwell & Woolgar 2000), the cut-off for prognostic purposes appears to be cohesive (pattern 1 and 2) and non-cohesive (pattern 3 and 4).

3.1.3.3. TNM staging:

Data of the pathological description of the tumors (tumor size, nodal status and distant metastasis) were derived from HPE report. The TNM staging was previously charted by the oral pathologist based on the International Union Against Cancer (Appendix 3).

3.2. Methods

3.2.1. Probe of FISH technique:

SO LSI Cyclin D1 DNA probe (Vysis, Inc. Downers Grove, IL, USA) which hybridizes to band 11q13 of human chromosome 11, was used. The centromeric probe for chromosome 11 (alpha satellite) was used for dual color FISH.

3.2.2. Analysis of chromosome copy number:

The pathologist selected areas for analysis by comparing hybridized slides to a corresponding H&E stained section. The hybridized signals appear as small spot since the region of a chromosome occupies only a small region of the nucleus. At least 200 nuclei were scored using a 100X objective in each defined histological area, and each nucleus was assessed for the chromosome copy number. If the signals ratio of the orange signals to the green signals is 2 or more than 2 then it is considered positively amplified.

Scoring criteria as follows:

1. Cytoplasmic materials should not cover the nuclei.
2. No overlapping observed between the nuclei.

3. Minor hybridized spots, which can be recognized as smaller size and lower intensity was excluded.
4. Signals can be counted on well separated signal spot.

3.2.3. Specimen processing:

Three μm thick sections were incubated at 37°C overnight and deparaffinized by washing in xylene rehydrated in graded ethanol and distilled water. After incubation in 0.2 M HCl at room temperature for 20 minutes, they were heat-pretreated in citrate buffer (2 × SSC, pH 7.0) at 80°C for 30 minutes. They were then digested with protease buffer at 37°C for 80 minutes, rinsed in 2 × SSC at room temperature for 3 minutes and dehydrated in graded ethanol (70, 85, and 100%) for 2 minutes each. For each slide, 1-μl of probe was mixed with 2-μl purified H2O and 7μl LSI hybridization buffer and applied to the dry slide, the tissue area was coverslipped and sealed with rubber cement. The slides were then incubated in a moist chamber (Hybridizer Instrument for in situ hybridization, DAKO, S2450, Denmark) for denaturation at 82°C for 5 minutes and hybridization at 37°C for about 16 hours.

The following day post hybridization washes were performed in 0.4 × SSC/0.3% NP -40 at 73°C for 2 minutes to remove non-specifically bound probe and in 2 × SSC/0.1% NP-40 at room temperature for 2 minutes and after application of 5 μL of mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI), the tissue area was coverslipped. These slides were viewed under a fluorescence microscope (BX 16, OLYMPUS, Tokyo, Japan).

3.2.4. Principle:

Target DNA, after fixation, is treated with heat to denature the double-stranded DNA, rending it single-stranded. The target DNA is thus available for annealing to a similarly denatures, single-stranded, fluorescently labeled DNA probe which has completely sequence. Following hybridization, unbound and non-specifically bound DNA probe is removed by s series of stringent washes and the DNA counterstained for visualization. Fluorescent microscopy then follows the visualization of the hybridized probe on the target material.

3.3. Statistical analysis

Categorical data was statistically analyzed by Chi-square and Fisher's exact test. Survival curves were constructed using the Kaplan-Meier method and compared using the log rank test. The level of significance was set at p less than 0.05. All statistical analyses were performed using SPSS 18 software (SPSS, Chicago, IL).

3.4. Expected output:

The finding may indicate the possibility of Cyclin D1 as a prognostic marker and therefore may predict the outcome of the patients treated for OSCC.