CHAPTER 3

Materials and Methods

3.1 Immunohistochemical study

3.1.1 Sample selection

Study group

The study design was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University (#54/2014). Thirty-four formalin-fixed and paraffin-embedded tissue sections between 2002 and 2012 that were histologically diagnosed as OSCC (*n*=34) were retrieved from the tissue archive of Oral Pathology, Department of Oral Biology and Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University under an approval of the dental hospital.

Control group

Ten normal-looking and non-inflamed oral mucosal tissues (*n*=10) were obtained from gingival biopsies overlying completely bony impaction of third molars from healthy volunteers, who underwent a surgical removal procedure at the Oral and Maxillofacial Surgery clinic, Faculty of Dentistry, Chiang Mai University. Informed consent was obtained from these volunteers prior to tissue collection.

The inclusion criteria for the control group was a healthy volunteer, who was 18 years old or older and had non-inflamed oral mucosa at the biopsy area. The volunteer who had underlying systemic diseases that prevented him or her from receiving the surgical removal of third molars, had oral mucosal lesions at the biopsy area, and refused to participate in this study was excluded from this study.

3.1.2 Data collection

Demographic data of patients, including age and sex, the location of OSCC, and the histologic grading (well-, moderately- and poorly-differentiated) were gathered from the histopathological reports and re-confirmed by a Board-certified oral pathologist.

3.1.3 Determination of ADAM9 expression in OSCC by immunohistochemistry

3.1.3.1 Formalin-fixed and paraffin-embedded tissue sections

1. Normal gingival biopsies from healthy volunteers were fixed in formalin and embedded in paraffin blocks according to a standard procedure.

2. Glass slides used in an immunohistochemical staining were first cleaned by immersion in 95% ethanol, treated with subbing solution and left to air dry.

3. Tissue sections at five-micron thickness from each paraffin block (either OSCC or normal oral mucosa) were applied onto the silanized glass slides.

4. The sections were de-paraffinized in xylene with two changes for 5 minutes each, and then rehydrated gradually through graded alcohols (100% ethanol and 70% ethanol) for 3 minutes each and distilled water for 3 minutes.

5. Endogenous peroxidase was blocked by incubating the sections for 10 minutes in 3% hydrogen peroxide in distilled water. After blocking the endogenous peroxidase activity, the sections were washed in distilled water for 5 minutes.

6. Antigen unmasking was performed by incubating the sections in sodium citrate buffer pH.6 at 100ºC for 15 minutes. The sections were left at room temperature for 20 minutes and washed in Tris-buffered saline (TBS) for 5 minutes.

3.1.3.2 Immunoperoxidase staining

1. The sections were blocked in 1.5% normal blocking serum (ImmunoCruz™ ABC Staining Systems, Santa Cruz Biotechnology, Santa Cruz, California, USA) in TBS for 20 minutes at room temperature.

2. Thereafter, a 100-µl quantity of diluted goat polyclonal antibody specific for ADAM9 (Santa Cruz Biotechnology) in TBS (1:100) was added on the sections at 4ºC overnight. For a negative control, the sections were incubated in normal blocking serum without the addition of primary antibody.

3. On the following day, the sections were washed in TBS for 5 minutes. A 100-µl quantity of diluted secondary anti-goat antibody (ImmunoCruz™ ABC Staining

Systems) in TBS (1:100) was added on the sections for 20 minutes at room temperature, and the sections were washed in TBS for 5 minutes.

4. Then, avidin-biotinylated horseradish peroxidase (ImmunoCruz™ ABC Staining Systems) was added on the sections for 20 minutes at room temperature, and the sections were washed in TBS for 5 minutes.

5. The color was developed by using 3, 3'-diaminobenzidine (Vector Laboratories, Burlingame, California, USA) for 3 minutes.

6. The sections were counter-stained in hematoxylin solution for 30 seconds and immediately washed with several changes of distilled water. The sections were left to air dry, mounted with 1–2 drops of glycerine gelatin mounting medium, covered with a glass coverslip, and observed under a light microscope. The images were captured by a digital camera (DP70, Olympus, Tokyo, Japan) attached to a desktop computer.

3.1.3.3 Immunohistochemical (IHC) staining scores

The stained sections were scored by two independent observers with kappa values of intra-observer calibration and inter-observer calibration equal to 0.908 and 0.848, respectively. The observers were blinded to clinicopathological information of each specimen. Scoring was performed by using ImageJ program (National Institutes of Health, Bethesda, Maryland, USA). Before scoring, each specimen was observed under 100x magnification power for determining the orientation of epithelium and connective tissue, and scoring was performed under 400x magnification power. In normal tissue, scoring was performed only in the epithelial layer, while in OSCC scoring was performed only in the epithelial cell nest in the connective tissue. Each section was first screened and selected for three fields of vision that represented the characteristic of each individual specimen. Cells that had any brown stain in the cytoplasm or on the cell membrane were considered positive regardless of the staining intensity.[55] The staining intensities were scored in four categories as 0 = no staining, 1 = weak, 2 = moderate and 3 = intense (Fig. 3.1).[56] The positive cells were counted and the percentage of positive cells was calculated by dividing with the total number of cells in each field of vision. Then, the mean percentage of positive cells in each section was determined from an average of three fields of vision. Finally, the immunohistochemical (IHC) score (0-3) of each tissue section was calculated by multiplying the mean percentage of positive cells with the intensity score (0-3).[57]

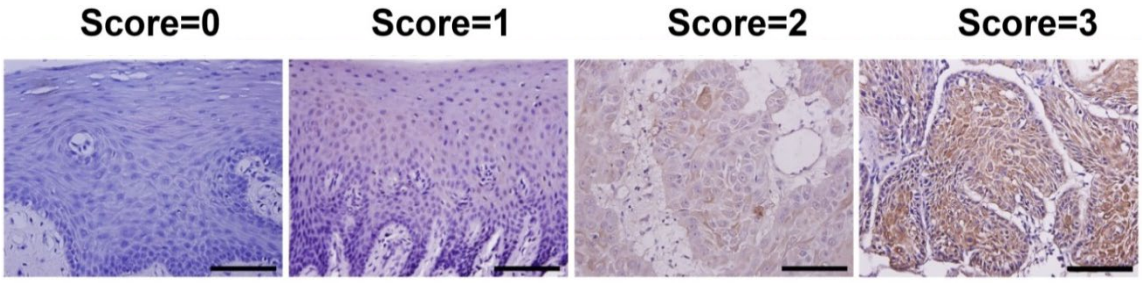

Figure 3.1 Four intensity scores: 0 = no staining, 1 = weak (light brown staining, visible only with high magnification), 2 = moderate (between 1 and 3), and 3 = intense (dark brown staining, visible with low magnification).[56] Bars = 100 micron.

3.2 *In vitro* studies

3.2.1 Cell lines and culture conditions

Four oral squamous cell carcinoma cell lines used in this study were HN5, HN6, HN15 and HN008.[58] Primary human oral keratinocytes (HOKs) were isolated from non-inflamed oral tissues overlying impacted third molars of eight healthy and non-smoking donors (n=8) with their informed consent as previously described.[59] As a positive control cell line for membrane ADAM9 expression, a hepatocellular carcinoma cell line, HepG2, was obtained from Professor Dr. Prachya Kongtawelert, Excellence Center of Tissue Engineering and Stem Cells, Department of Biochemistry, Faculty of Medicine, Chiang Mai University. HOKs were cultured in serum-free keratinocyte growth medium (KGM; Lonza, Walkersville, Maryland, USA), whereas four oral cancer cell lines and HepG2 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen™, Carlsbad, California, USA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen™). All cells were maintained at 37ºC in a humidified chamber with 5% CO_2. Culture medium was changed every two days until cell growth reached 80% confluence. The cells were then collected by treatment with EDTA and cell scraping for flow cytometry, whereas their total protein contents were extracted in radio-immunoprecipitation assay (RIPA) buffer for Western blot hybridization. In addition, the serum-free KGM was switched to KGM containing various calcium concentrations, including 0.03, 0.15 and 1.2 mM, at 80% HOK confluence for 48 hours in order to compare ADAM9 expression between undifferentiated and differentiated HOKs.

3.2.2 Indirect immunofluorescence staining and flow cytometry

Four cancer cell lines and HepG2 were washed with phosphate-buffered saline (PBS) twice, and incubated in 3 ml quantity of 0.5 mM EDTA pH 7.3 at 37°C for 3 minutes. Cells were removed from 100-mm culture dishes by scraping with cell scrapers, centrifuged at 400g for 5 minutes, and then washed twice with PBS pH 7.2. To block nonspecific Fc-receptor-mediated binding of antibodies, cells were pre-incubated with 10% AB serum in 2% sodium azide (NaN_3) at 4 ºC for 1 hour. Note that the quantity of AB serum used in each cell sample varied in order to obtain the cell density at $2x10^6$ cells per µl. Fifty microliters of blocked cells were then transferred to v-bottom 96-well plates and incubated for 30 minutes at 4°C with 200 µl of the anti-ADAM9 antibody (LifeSpan BioSciences, Inc., Seattle, Washington, USA) in FACS buffer (1:20). As two negative controls, cells were incubated with either 200 µl of the rabbit immunoglobulins (20 µg/ml), normal rabbit serum that was purified by a protein G column as a control antibody, or 200 µl of the FACS buffer as a conjugate control. The cells were washed with a 200-µl quantity of FACS buffer and centrifuged at 718g for 5 minutes three times. After that, the cells were incubated with phycoerythrin (PE)-conjugated sheep $F(ab')_2$ anti-rabbit antibody (1:1000) (Invitrogen™) and 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich, Zwijndrecht, Netherlands), a dead cell marker used to gate dead cells out of flow cytometric analysis, at 4°C for 30 minutes. The cells were again washed with a 200-µl quantity of FACS buffer and centrifuged at 718g for 5 minutes three times. The fluorescent signals were detected by a flow cytometer (FACSCalibur, Becton Dickinson, Sunnyvale, California, USA) and analyzed by FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

3.2.3 Isolation of total protein and Western blot hybridization

Whole cell lysates of all cell lines were extracted in RIPA buffer.[60] The quantity of total protein in the whole cell lysates was determined by the BCA Protein Assay kit (Pierce®, Rockford, Illinois, USA) according to the manufacturer's protocol. Each whole cell lysate sample was denatured by heating at 100ºC for 5 minutes. A 20-µg quantity of whole cell lysates was resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California, USA). Following transfer, unoccupied binding sites on the membranes were blocked with 5% non-fat dry milk (Santa Cruz Biotechnology)

at room temperature for 1 hour. Then, the membranes were incubated with the primary antibody against ADAM9 (1:500), filaggrin (1:500), a late differentiation marker of HOKs, or β-actin (1:1000) as a housekeeping gene control (Santa Cruz Biotechnology) at 4ºC overnight. On the following day, the membranes were incubated with either anti-mouse (for the mouse monoclonal antibodies specific for filaggrin and β-actin) or anti-goat (for the goat polyclonal antibody specific for ADAM9) secondary antibody (Santa Cruz Biotechnology) at 1:2000 for 1 hour at room temperature. The LumiGLO Reserve chemiluminescent reagent (KPL, Gaithersburg, MD, USA) was used as a substrate, and the signal was captured by a charge-coupled device (CCD) camera attached to the ChemiDoc XRS gel documentation system (Bio-Rad Laboratories). Some digitized images were analyzed by Scion image program (Scion Corporation, Maryland, USA) to quantify the intensity of ADAM9 bands from Western blot hybridization. The ADAM9 expression was normalized by β-actin expression in each corresponding sample, and adjusted to the percentage of ADAM9 expression by comparing with the normalized ADAM9 expression in HN008, set to 100%.

3.3. Statistical analysis

The Mann-Whitney *U* test was used to compare the IHC scores, representing ADAM9 expression, between OSCC and normal oral tissues. The Spearman correlation coefficient was used to determine the correlations between the IHC scores and the histologic grading. The Independent sample *t*-test was used to compare the percentages of ADAM9 expression between oral cancer cell lines and normal HOKs. The statistical analyses were carried out by the Statistical Package for Social Sciences version 17 (SPSS, Inc., Chicago, Illinois, USA). P-values less than 0.05 were considered statistically significant.