Chapter 3: Materials and Methods

3.1 Study design

This is a descriptive study to validate the expressions of GNA12 and IFITM3 in oral cancer using frozen and formalin-fixed paraffin embedded tissues selected by convenience sampling. The functional roles of GNA12 and IFITM3 were further determined in oral cancer cell lines by *in-vitro* cell culture assays (Appendix I).

3.2 Clinical specimens and cell lines

The use of clinical specimens in this study has been approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [MEC approval number: DF OP0703/0017]. Socio demographic and clinical information of the examined cases were obtained from the MOCDTBS, Oral Cancer Research and Coordinating Center (OCRCC). The following are the specimen types used in this study.

3.2.1 Frozen tissues

Frozen tissues were from OCRCC and Cancer Research Initiatives Foundation (CARIF). A total of 47 OSCC tissues were examined in this study. The 18 oral mucosa tissues consist of 8 non-malignant tissues from oral cancer patients and 10 from the flap of gum removed from individuals during wisdom tooth extraction. Although some of these gum epithelium tissues are hyperplastic, they were considered genetically normal and hence used as control (Saleh et al., 2010). All these tissues were used as a source to obtain RNA for complementary DNA (cDNA) synthesis and subsequent quantification of target gene expression by quantitative PCR (QPCR) analysis. The list of frozen specimens used is attached in Appendix II.

Inclusion criteria:

1. All primary tumours of OSCC
2. Tissues having ≥ 70% tumour cellularity.
3. Tissues with RNA integrity number (RIN) ≥ 4.5.

Exclusion criteria:

1. Dysplastic epithelial and connective tissues.
2. Specimens with inadequate clinical information.

3.2.2 Formalin-fixed paraffin embedded tissues (FFPE)

FFPE tissues were from the Department of Oral Pathology, Oral Medicine and Periodontology (OPOMP) and the Oral Pathology Diagnostic Laboratory of Faculty of Dentistry, University of Malaya. A total of 67 FFPE oral tissues (44 OSCC and 23 non-malignant oral tissues) were constructed into tissue macro-arrays (TMaA). Oral mucosa tissues comprised of 10 fibro epithelial polyp (FEP) and 13 epithelium of the gum excised from individuals who had their wisdom tooth extracted. These oral mucosa tissues are non-malignant and were genetically similar to those of normal tissues, hence were used as controls (Saleh et al., 2010). Each TMaA block consists of at least 3 tumours and 1 non-malignant oral tissue. In order to construct the TMaA blocks, hematoxylin and eosin (H&E) staining of the FFPE tissue blocks were used as reference to identify the tissue area with more than 70% epithelial cells. The entire surface epithelium until the underlying stromal area was excised by a scalpel and transferred to a recipient paraffin block, which was then embedded in wax. TMaA blocks were sectioned and subsequently used for H&E and immunohistochemistry (IHC) staining. The list of FFPE specimens used is attached in Appendix III.

Inclusion criteria:

1. Tissues of primary tumour
2. Tissues having ≥ 70% tumour cellularity

Exclusion criteria:

1. Dysplastic epithelial and connective tissues.
2. Specimens with inadequate clinical information.

3.2.3 Oral cell lines

Oral cell lines established from Malaysian oral cancer patients were provided by CARIF (Hamid et al., 2007). All the 15 OSCC cell lines and 2 non-malignant primary cultures were cultured for RNA extraction, protein extraction, and *in-vitro* studies. Cell lines information were summarized in Appendix IV.

Inclusion criteria: Oral cell lines derived from the primary site of the tumour

Exclusion criteria: Oral cancer cell lines from recurrent tumour

3.3 Validation of GNA12 and IFITM3 mRNA expression in oral cancer tissues

3.3.1 RNA isolation from frozen tissues

Frozen tissues were retrieved from liquid nitrogen storage and embedded in Jung tissue freezing medium (Leica, Heidelberger, Germany) to section for histological evaluation by an oral pathologist. When there is ≥70% tumour cells in the tumour, 20x5 µm cryosections were harvested, and RNA extraction was performed using the RNeasy micro kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendation. A detail of the extraction method is included in Appendix V.

The quality and concentration of RNA was determined using RNA 6000 Nano kit and Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The RNA Nano chip was set up on the chip priming station following manufacturer's protocol. On the RNA 6000 Nano chip, all wells were loaded with 9µl of gel-dye mix, followed by 5

µl of marker. RNA samples of 1 µl were heat denatured at 70ºC for 2 min, and loaded into the respective well on the chip. Denatured RNA ladder was also loaded into the designated well. The chip was vortexed for 1 min at 2400 rpm on the IKA Vortex mixer and then ran on the bioanalyzer to determine the concentration and RIN value.

3.3.2 cDNA synthesis

Total RNA isolated from frozen tissue sections were reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Briefly, 50 µl of sample consisting 2 µg of total RNA, was added to 50 µl of 2x RT master mix (10x Reverse Transcription Buffer, 25x dNTPs, 10x random primers, 50 U/µl MultiScribe™ Reverse Transcriptase and RNase-free water). Reverse transcription was done in a thermal cycler at 25ºC for 10 min followed by 37ºC for 120 min.

3.3.3 QPCR analysis for the quantification of mRNA levels of target genes

Primers for QPCR were designed by Primer Express 2.0 software (Applied Biosystem, CA, USA) and their alignments to the respective mRNA sequences were determined using BLAST (http://blast.ncbi.nlm.nih.gov). Primers were synthesized by Sigma-Proligo (Singapore) and sequences are listed in Table 3.1. For quality control, PCR efficiency of the primers was determined to be at least 97%.

The relative abundance of GNA12 and IFITM3 mRNA were assessed using Power SYBR Green assay on the ABI Prism Sequence Detector 7000 (Applied Biosystem, CA, USA). QPCR was performed in triplicates in a total volume of 25 µl, containing Power SYBR Green master mix, 750 nM forward and reverse primers and 20 ng of cDNA. Reaction was carried out using cycling conditions with 10 min of polymerase activation at 95ºC, and 50 cycles at 95ºC for 15 sec and 60ºC for 1 min. Relative quantification was performed by comparative Ct method to calculate the level

of GNA12 and IFITM3 mRNA expressions in relative to the housekeeping gene GAPDH. Expression of target genes in the tumour was subsequently calibrated against oral mucosa controls and expressed as relative quantification (RQ) value, as calculated by the ABI Prism Sequence Detector 7000 software. The RQ value of 2 or more was considered as over-expression. A dissociation protocol was performed to check for primer-dimer formation and unspecific amplicons.

Table 3.1: Primers sequence for QPCR

GNA12	Fw: 5' ATAAGTCAGATTGTTAACTCCAAGATTGA 3' Rv: 5' AGCCAGACCCTCCCAATGTT 3'
IFITM3	Fw: 5' GACCATTCTGCTCATCGTCATC 3' Rv: 5' GCACTTTATTGAATGCCATTGTAGA 3'
GAPDH	Fw: 5' GAAGGTGAAGGTCGCAGT 3' Rv: 5' GAAGATGGTGATGGGATTTC 3'

3.4 Validation of GNA12 and IFITM3 protein expression in oral cancer tissues

3.4.1 Immunohistochemistry staining

Immunohistochemistry analysis for GNA12 and IFITM3 was performed on TMaA tissues. Paraffin-embedded TMaA tissue sections were dewaxed at 65ºC and gradually rehydrated in descending grades of ethanol (100%, 95% and 70%) and finally in water. Antigen retrieval was done by boiling the tissue sections in citrate buffer (pH6.0) for 10 min in a microwave, and cooled for 20 min to enable the antigenic site to reform. Tissues sections were subjected to endogenous enzyme block for 10 min using the peroxidase blocking reagent (Dakocytomation) in a humidified chamber. Polyclonal primary antibodies against GNA12 (Santa-Cruz, sc-409) and IFITM3 (Abcam, ab15592) were used in 1:75 dilutions. Tissues sections were incubated with primary antibodies for 90 min.

Detection was carried out using Dako REAL™ Envision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse kit (Dako, Kyoto, Japan), and staining was developed by 3,3'-diaminobenzidine (DAB) with observation under a microscope. Brown staining

was considered positive. Tissues were counterstained with hematoxylin for 10 sec, and dehydrated in increasingly concentrated ethanol (70%, 95% and 100%) and finally in xylene before mounting with DPX mountant (Fluka, Steinheim Germany). Human breast cancer tissue and colorectal cancer tissue were used as positive control for GNA12 and IFITM3, respectively (Appendix VI). For negative control, tissues were incubated with PBS instead of primary antibody, and processed as mentioned above.

3.4.2 IHC staining evaluation

Tissue sections were examined under light microscopy at 100x magnification in three random fields and staining was evaluated in the epithelial cells by semi-quantitative method. Expression of target proteins were interpreted based on the relative signal intensity and scored as following: 0 for negative, +1 for weak positive, +2 for moderate and +3 for strong positive (Yamamoto et al., 2001). If the staining intensity was heterogeneous, then scoring was based on the greatest degree of intensity (Bhargava et al., 2006). All tissue sections were graded by two independent investigators and results were compared. The percentage of stained cells was also recorded. Discrepancies in the interpretations were discussed and tissues were reviewed in additional 3 random microscopic fields to achieve consensus. Finally, GNA12 and IFITM3 protein expressions were further categorized into 2 groups: low (score 0 and +1) and high expressors (score +2 and +3) for statistical analysis.

3.5 Maintenance of cell lines

Oral cancer cell lines were maintained in DMEM/F12 complete medium, as reported by (Hamid et al., 2007). Packaging cells (293FT and GP-293) were grown in complete DMEM high-glucose complete medium, as instructed by the manufacturers. Details of the tissue culture conditions are included in appendix VII.

3.6 Exogenous expression of target genes in oral cancer cell lines

Oral cancer cell lines with low levels of endogenous GNA12 and/or IFITM3 were predetermined by QPCR and Western blot (Appendix X). The selected cell lines were engineered to exogenously express the target genes using retroviral or lentiviral expression vectors.

3.6.1 Plasmid preparation and propagation

(A) GNA12 retroviral expression plasmid

Retroviral expression vector pLXRN (Appendix XI) and pLXRN/Gα12QL were obtained from Prof. Patrick Casey, Duke University Medical Centre. The Gα12QL insert has a point mutation resulting in the change of the conserved glutamine to leucine at amino acid position 231. This mutation causes the intrinsic GTPase activity of GNA12 to be greatly impaired resulting in the constitutive activation of GNA12 because it is predominantly in the GTP-bound form. Plasmids were propagated by transformation into XL1-Blue *E. coli* competent cells via $CaCl_2$ method. Details of the transformation procedures are illustrated in appendix VIII.

Plasmids were extracted using HiSpeed plasmid MIDI kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Plasmids were eluted with 500 µl sterile water and quantitated on the NanoDrop. To ensure that these plasmids are of the right size, 0.7% agarose gel electrophoresis was carried out along with a supercoiled DNA ladder (Promega, WI, USA). The Gα12QL insert was confirmed by sequencing using MMLV-Psifor and RSV-Prorev vector primers in Table 3.2.

Table 3.2: Primers sequence for pLXRN vector primers

MMLV-Psifor	5' GTCTGAATTTTTGCTTTCG 3'
RSV-Prorev	5' CTCATCGTTACCATGTTGC 3'

(B) IFITM3 lentiviral expression vector

In order to clone IFITM3 into lentiviral expression vector pLenti6.3 (Appendix XII), full length coding region of IFITM3 was amplified by PCR using primers designed by Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast) as listed in Table 3.3. PCR mix consisting 1x buffer, 1.5 mM $MgCl_2$, 0.2 μM IFITM3 forward and reverse primers, 0.2 mM dNTPs and 1.5U GoTaq polymerase (Promega, WI, USA) was added to 50 ng of cDNA from ORL207 cells that showed high IFITM3 mRNA level (Appendix X). The PCR reaction was performed in a total volume of 50 ul with cycling condition as below:

Step	Temperature	Time	Number of cycles
Initial denaturation	95ºC	2min	1
Denaturation	95ºC	30sec	
Annealing	58ºC	30sec	30
Extension	72ºC	30sec	
Final Extension	72ºC	7min	1
Soak	4ºC	Indefinite	1

PCR product was electrophoresed on 2% agarose gel at 100V for 25 min to ensure that the product is of the right size, and further purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany). Approximately 45 ng of purified IFITM3 was cloned into 10 ng of pre-linearized lentiviral expression vector pLENTI6.3 with single 3'-thymidine overhang by topoisomerase method according to the manufacturer's instructions (Invitrogen, CA, USA). Following this, the ligated product was immediately transformed into Stbl3 *E.coli* competent cells by heat shock method. Cells were screened on LB plate containing 100 μg/ml ampicillin and several clones were cultured and further subjected to culture PCR using the IFITM3 forward primer and V5 reverse primer to confirm that the insert was cloned in the correct orientation and in frame with V5 epitope. The PCR reaction and cycling conditions were carried out as mentioned above. Inserts were sequenced using CMV and V5 primers to ensure no mutations are present.

These plasmids were further propagated for extraction using HiSpeed plasmid MIDI kit (Qiagen, Hilden, Germany), and the use in subsequent mammalian cell culture studies, while transformed Stbl3 cells were cryopreserved in 15% glycerol.

Table 3.3: Primers used for cloning IFITM3 into pLenti6.3

Primers	Sequence
IFITM3 forward primer	5'GCCATGAATCACACTGTCCAA 3'
IFITM3 reverse primer	5' TATCCATAGGCCTGGAAGATCAG3'
CMV forward primer	5'CGCAAATGGGCGGTAGGCGTG3'
V5 reverse primer	5'ACCGAGGAGAGGGTTAGGGAT3'

3.6.2 Virus transduction to over-express GNA12 in oral cancer cells

(A) Producing retrovirus in GP-293

The pLXRN/Gα12QL plasmid was transfected into GP-293 cells using cationic lipid method. GP-293 cells were seeded at a density of 5.5 E5/ml in a 60 mm dish 18 hours prior to transfection by lipofectamine 2000 (Invitrogen, CA, USA). Approximately 5 µg of pLXRN/Gα12QL and 5 µg of pVSVG envelope plasmids were mixed with 60 µl of lipofectamine 2000 in DMEM high glucose medium without serum. The DNA-lipofectamine complexes formed after 20 min of incubation at room temperature was added to the GP-293 cells and further incubated in tissue culture incubator for 24 hours. Culture medium was replaced with DMEM/F12 complete medium and cells were incubated for another 48 hours to allow viral titers to increase. Viruses present in the culture medium was collected and filtered through a 0.45 µm cellulose acetate filter to remove cell debris. Then, these viruses were used to deliver GNA12 into oral cancer cell line and the remaining viruses were aliquoted and stored at -80ºC. Viruses carrying pLXRN empty vector was also produced as mentioned above, and used as control.

(B) Retrovirus transduction and analysis

Viruses carrying Gα12QL were transduced into oral cancer cell line ORL150 which has low endogenous expression of GNA12 (Appendix X). Briefly, 1 ml of virus media prepared from section 3.6.2(A) was mixed with 2 ml DMEM/F-12 complete medium supplemented with 10 μg/ml polybrene, and then added onto ORL150 cells seeded 24 hours prior to transduction at a density of 1 E5 cells per 60 mm dish. Cells were incubated for 24 hours in the CO_2 incubator. The following day, the medium was replaced with fresh DMEM/F-12 complete medium and further cultured for 48 hours. ORL-150 cells were subjected to a second infection by repeating the steps mentioned above. The transformed ORL-150/Gα12QL cells were maintained in DMEM/F-12 medium containing 75 μg/ml G418 antibiotic for the selection of cells stably expressing activated form of GNA12. Similarly, a different plate of ORL150 cells was also transduced with viruses carrying empty pLXRN vector for used as control.

3.6.3 Virus transduction to over-express IFITM3 in oral cancer cells

(A) Producing lentivirus in 293FT

Viruses carrying IFITM3 was produced in similar manner to that used for GNA12. Briefly, 293FT cells were seeded at 5 E6 in 10 ml DMEM-high glucose complete medium 24 hours prior to transfection. The next day, 3 μg of pLenti6.3/IFITM3 and 9 ug of the ViraPower packaging mix were co-transfected into 293FT cells using 36 μl of lipofectamine 2000, and subsequent procedures are carried out as described in section 3.6.2 (A). The pLenti6.3/lacZ control vector was also transfected for use as control.

(B) Lentivirus transduction and analysis

ORL188 cells were seeded at 4 E5 cells in 5ml DMEM/F12 complete medium 24 hours prior to infection. IFITM3 was delivered into ORL188 using 1 ml of lentivirus produced

in section 3.6.3 (A) and 2 ml of DMEM/F-12 complete medium containing 10 μg/ml polybrene. These cells were incubated for 72 hours in the CO_2 incubator. ORL-188 cells were subjected to a second infection by repeating the steps mentioned above. Transformed ORL-188/IFITM3 cells were cultured and maintained in DMEM/F-12 medium containing 7.5 μg/ml blasticidin for the selection of cells stably expressing IFITM3. ORL188 cells were also transduced with viruses for pLENTI6.3 control vector.

3.6.4 Determining GNA12 and IFITM3 mRNA levels in transduced-cells

The increased expression of GNA12 and IFITM3 after virus transduction was determined by QPCR. RNA was harvested from cell culture at 70% confluence using Tri-Reagent (MRC, OH, USA), according to the manufacturer's protocol and details of this procedure can be found in appendix V. Total RNA from oral cell lines were converted into cDNA as described in section 3.3.2. Then, the mRNA levels of GNA12 and IFITM3 in these cells were evaluated by QPCR using the condition as described in section 3.3.3.

3.6.5 Determining GNA12 and IFITM3 protein expressions in transduced-cells

The protein levels of GNA12 and IFITM3 in the retro-/lenti- viral transduced cells were evaluated by Western blotting (refer to Appendix IX for detail procedure). Briefly, protein extraction and quantification was performed. Protein was heat-denatured in 6x Laemmli buffer and of 50 μg of protein was resolved in 12% SDS-PAGE. Subsequently, proteins were transferred onto 0.45 micron nitrocellulose membrane (Pierce, IL, USA) using the Mini Trans-Blot Electrophorectic Transfer Cell (Bio-Rad, CA, USA) in wet condition at 400 mA for 60 min. Membranes were blocked with 5% skimmed milk/TBST to prevent non-specific binding of antibodies to the membrane. Then, the following primary antibodies were used to probe the membrane: GNA12 at 1:200

dilution (sc-409, Santa Cruz, USA) and IFITM3 at 1:1000 dilution (H00010410-M01, Abnova, Taiwan). Detection of bands was done using secondary antibodies conjugated to IRDye at 1:10000 dilution (LICOR, Nebraska, USA), and scanned using the Odyssey scanner (LICOR, Nebraska, USA). The intensity of the protein bands were measured by densitometry using the Odyssey software (LICOR, Nebraska, USA). The anti-actin monoclonal antibody was used at 1:1000 dilution (MAB1501, Chemicon, USA) to control for loading variation.

3.7 Determination of the effects of GNA12 and IFITM3 expression in oral cancer cell lines by *in-vitro* functional assays

3.7.1 Cell proliferation assay

The effects of target genes expression on oral cancer cell proliferation was measured using the CASY cell counter (Innovatis AG, Reutlingen, Germany). Transformed oral cancer cell lines ORL150/pLXRN and ORL150/Gα12QL were seeded at 5 E4 cells/ml in DMEM/F12 complete medium and cultured in the CO_2 incubator for up to 9 days. Cell count was performed at every 24 hours time point, whereby cells were trypsinized, neutralized and harvested into a 15 ml tube. Then, 50 µl of the single cell suspension was transferred to a CASY cup containing 10 ml of CASYton electrolyte and further subjected for analysis in the cell counter based on electric current exclusion and pulse area analysis. Total cell and viable cell numbers were recorded. Cell count was measured 3 times by the CASY cell counter and the average reading was used for the generation of a growth curve. Cell doubling time was calculated based on the exponential phase of the growth curve and determined using the formulation as below:

$$ratio = \frac{cell\ no.\ on\ harvest\ day (4, 5\ or\ 6)}{cell\ no.\ seeded\ at\ day\ 0} \qquad cell\ doubling\ time = \frac{\ln 2}{\ln (ratio)} \times no\ of\ days$$

Similarly, the effects of IFITM3 over-expression on oral cancer cell growth was also assayed as mentioned above. The growth of ORL188/IFITM3 cells was compared to the control cells ORL188/pLENTI6.3.

3.7.2 Cell migration assays

(A) Monolayer scratch assay

Monolayer scratch assay was carried out to examine the effects of target gene expression on cell migration (Liang et al., 2007). Transformed-cells were seeded at 4 E5 cells/ml and cultured for 48 hours to form confluent monolayer. Cells were treated with 10 μg/ml mitomycin C for 2 hours to inhibit cell proliferation. A line was scratched through the cell monolayer using a P200 pipette tip by applying constant pressure to create an open wound. The cell monolayer was gently rinsed with 1x PBS to remove cell debris and images of the open wound were captured at 3 random fields under the microscope at 100x magnification. The cells were further cultured in DMEM/F12 complete medium for 18 hours. After that, cells were rinsed with 1x PBS and images of the open wound area after 18 hours were captured. The open wound areas of the transformed-cells were analyzed and compared to that of the control cells using T-scratch software (Geback et al., 2009)

(B) Transwell migration assay

Transformed-cells were also tested on Transwell migration assays using cell culture inserts with PET membrane of 8 μm pore size, according to the manufacturer's instructions (BD Biosciences, MA, USA). Briefly, cells were cultured until 70% confluence and serum-starved overnight. Cells were harvested by trypsinization, pelleted and re-suspended in 3 ml of serum-free DMEM/F-12 medium supplemented with 0.1% BSA at a concentration of 2 E5 cells/ml. In the 24-well plate, inserts were

placed into wells containing 500 µl of pre-filtered 3T3-conditioned media as chemo-attractant, and 2 E4 cells were seeded onto the inserts. The plate was incubated for 24 hours to enable the migration of cells through the membrane in respond to chemo-attractant. Non-migrated cells were scraped by cotton swab, while the migrated cells at the bottom of the membrane were fixed with 4% formaldehyde for 15 min and stained with 0.2% crystal violet for 10 min. Membranes were washed in water to remove excess stain and air-dried. Then, the membrane was excised from the insert housing and mounted onto microscope slide for observation under the microscope at 200x magnification. The number of stained cells were counted in 4 randomly chosen microscopic fields and averaged.

3.7.3 Matrigel invasion assay

The potential of transformed-cells to invade through the matrigel barrier was studied using the Biocoat Matrigel 24-well invasion chamber (BD Biosciences, MA, USA) according to the manufacturer's instruction. Experiments were performed in the same manner as described in section 3.7.2 (B), except that the inserts used were pre-coated with matrigel basement membrane matrix. The transformed-cells that invaded through the matrigel barrier were compared to the control cells.

3.7.4 Detection of apoptosis by flow cytometry

Effects of IFITM3 expression on cell apoptosis was evaluated by Annexin V staining and flow cytometry. Cells were seeded at 5 E4 cells/ml and cultured to 80% confluent. Floating and adherent cells were harvested and washed twice with cold PBS. Cells were resuspended in 1x binding buffer at a concentration of 1 E6 cells/ml, and 100 µl of the solution was transferred to a 5 ml tube for incubation with 2.5 µl of FITC Annexin V and 2.5 µl of propidium iodide (PI) for 15 min in dark at room temperature. Finally, 400

µl of 1x Binding Buffer was added to the tube and analyzed by flow cytometry (FACSCalibur, BD Biosciences, MA, USA). FITC Annexin V stains cells undergoing apoptosis by binding to the membrane phospholipid phosphatidylserine that was exposed to the environment upon cell death. PI was used to distinguish viable from non-viable cells. Thus, cells that stain positive for FITC Annexin V and negative for PI are undergoing apoptosis. The percentage of apoptosis population in IFITM3 transformed-cells was compared to the vector control cells as described above.

3.8 Statistical analysis

Statistical analyses were performed using SPSS software version 16.0 (SPSS Inc., IL, USA). Statistical significance was defined with p-value < 0.05.

The mRNA expression of GNA12 and IFITM3 obtained from RT-QPCR analysis was evaluated by Mann-Whitney test to determine the differences of mRNA level between tumour and non-malignant oral tissue specimens. GNA12 and IFITM3 proteins expressions assessed by IHC staining was evaluated by standard chi-square test to determine if high level of the target proteins are associated with tumour size, involvement of nodes invasion, tumour staging and tumour site.

Cell culture functional assays were conducted in triplicates and repeated at least 3 times. Results are presented as mean ± standard error. Assumption to conduct t-test was checked and the outcome was normally distributed (Appendix XIII). Independent t-test with two-tailed distribution was used to determine differential significance between the transformed-cells and the control cells.