CHAPTER II

MATERIALS AND METHODS

2.1 CLINICAL COHORT AND SPECIMENS

All 27 NPC and 3 non-NPC cDNAs subjected for real-time quantitative PCR (Section 2.4.3) were derived from samples obtained from the Tung Shin Hospital, Kuala Lumpur. For immunohistochemistry (Section 2.5.4), 43 NPC and 11 non-NPC paraffin-embedded tissues were obtained from the Tung Shin Hospital, Kuala Lumpur and NCI Hospital, Negeri Sembilan. Prior informed consent was obtained from patients. The histologic characteristics of the non-NPC biopsies used in immunohistochemistry are summarized in Table 2.1.

Table 2.1: Non-NPC samples used in immunohistochemistry

Code	Histology
NPC 12	Nasopolyps (noncancerous growth of the nasal linings)
NPC 16	Nasopharyngitis (inflammation of the nasopharynx)
NPC 21	Normal epithelium
NPC 48	Synovial sarcoma (a form of soft tissue cancer)
NPC 51	Nasopharyngitis
NPC 65	Nasopharyngitis
NPC 73	Nasopharyngitis
NPC 84	Nasopharyngitis
NPC 88	Adenoid (mass of lymphoid tissue)
NPC 98	Nasopharyngitis
NPC 99	Lymphoid hyperplasia (enlarged lymph tissue), rhinosinusitis (inflammation of the nose tissue)

2.2 CELL LINES

NPC and immortalized nasopharyngeal epithelial cell lines used in this study, as well as their EBV status are summarized in Table 2.2. Cell lines were gifts from Professor SW Tsao (Department of Anatomy, University of Hong Kong, Hong Kong, China).

2.3 CELL CULTURE

2.3.1 Maintenance of cell lines

NPC cell lines CNE1, HONE1, HK1, SUNE1, TWO1, TWO4 cells were cultured in RPMI 1640 (Gibco, Invitrogen, USA) containing 10% heat-inactivated FBS (Gibco, Invitrogen, USA). Immortalized nasopharyngeal epithelium NP69 cells were maintained in defined KSFM (Invitrogen, USA) supplemented with 5% FBS, 0.2μg/ml recombinant EGF (Invitrogen, USA), and 25μg/ml bovine pituitary extract (Invitrogen, USA). NP460 cells were cultured in 1:1 ratio of defined KSFM (Invitrogen, USA) and EpiLife (Invitrogen, USA). All cultures were maintained in a humidified chamber with 95% air and 5% CO_2 at 37°C and periodically checked for mycoplasm contamination (VenorGeM, Germany). Medium was changed thrice a week.

Table 2.2: Cell lines

Cell line	Origin	EBV status	Reference
CNE1	Differentiated NPC	Negative	("Establishment of an epitheloid cell line and a fusiform cell line from a patient with nasopharyngeal carcinoma," 1978)
HK1	Differentiated NPC, recurrent	Negative	(Huang et al., 1980)
HONE1	Undifferentiated NPC	Negative	(Glaser et al., 1989)
SUNE1	Undifferentiated NPC	Negative	(Chen et al., 1998)
TWO1	Differentiated NPC	Negative	(Lin et al., 1990)
TWO4	Undifferentiated NPC	Negative	(Lin et al., 1993)
NP460	Non-malignant nasopharyngeal epithelium, immortalized using hTERT	Negative	(Li et al., 2006)
NP69	Non-malignant nasopharyngeal epithelium, immortalized using SV40T	Negative	(Tsao et al., 2002)

2.3.2 Trypsinization of Cell Lines

Cells were passaged when they were about 70% in confluency. The culture medium was aspirated and the cells were rinsed once with PBS. The cells were detached by incubating with Trypsin-EDTA (Gibco, Invitrogen, USA) for 5min. An equal volume of 10% RPMI media was added to neutralize the enzymatic action. The cell suspension was centrifuged at *300 x g* for 5min and resuspended in the growth media. Cell count was done using CASY® Model TT cell counter (Roche Innovatis AG, Germany).

2.3.3 Cryopreservation of Cell Lines

$1x10^6$ cells were resuspended in 1ml 10% RPMI containing 10% v/v DMSO for all NPC cell lines, or in FBS containing 10% DMSO for normal immortalized cell lines. Gradual freezing of the cells were achieved by storing in MrFrosty™ Cryo 1°C Freezing Container (Nalgene®, USA) in -80°C overnight before storing in liquid nitrogen.

2.4 MOLECULAR BIOLOGY

2.4.1 Total RNA Isolation

Total RNA was extracted from cultured cells that are at about 70% in confluency using Nucleospin® RNA II (Macherey-Nagel, Germany) according to manufacturer's protocol. RNA concentration was measured using NanoDrop ND-1000 spectrophometer (NanoDrop Technologies, Thermo Scientific, USA). 1µg RNA was electrophoresed on 1% agarose gel containing trace amount of 10mg/ml ethidium bromide. The gel was viewed under ultraviolet (UV) rays using ChemImager (AlphaInnotech, USA). Two distinct bands of 28S and 18S ribosomal RNAs in a ratio of 2:1 indicate intact RNA.

2.4.2 First-strand cDNA Synthesis

A 12µl reaction mixture containing 1µl 10mM dNTP Mix (Promega, USA), 1µl OligodT primers (Promega, USA), 1µg total RNA and RNAse-free water was heated at 65°C for 5min. Subsequently, 4µl of 5X First-Strand Buffer plus 2µl of 0.1M DTT were added to the reaction mixture. After incubation for 2min at 42°C, 1µl of 200U SuperScript II reverse transcriptase was added. The reaction was continued for another 50min at 42°C before inactivation at 70°C for 15min. The reaction was performed in a 500µl microcentrifuge tube using GeneAmp PCR System (Applied Biosystems, USA).

2.4.3 Real-time Quantitative PCR (qPCR)

Gene-specific primers were designed using Primer Express 2.0 software (Applied Biosystems, USA) and synthesized by Sigma-Proligo (Singapore). Specificity of the primer pairs to the target transcript was verified by aligning to their respective mRNA sequences using BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). Primer sequences are listed in Table 2.3.

qPCR was performed using ABI Prism® 7000 Sequence Detection System (Applied Biosystems, USA). The reaction mixture contained 100ng first-strand cDNA, 7.5µl water, 12.5µl SYBR® Green PCR Master Mix (Applied Biosystems, USA) and 2µl each of 10µM forward and reverse gene-specific primers (Sigma-Proligo, Singapore). The thermal cycling conditions were as follows: 10min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1min at 60°C. In parallel, GAPDH was also amplified to serve as an internal control. Experiments were performed in triplicate for each reaction. A dissociation protocol was performed to confirm the specific amplification of the PCR products. In addition, the PCR products were electrophoresed on 2% agarose gel. Relative expression RQ was determined as $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ (sample) – ΔCT (calibrator). ΔCT means CT(target gene) – CT(GAPDH), where CT is the threshold cycle value.

Table 2.3: Primers Used in PCR

Target	Forward and Reverse Primer	Product Size (bp)
GAPDH	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	226
FJX1	CCCGCAAAGGTGTCTAAAAACT GTGCTGGCACAGTAAAGAATCCT	162
WNT5A	CATTATGGGCTCAAATAGAAAGAAGA AAAGAGCTAGGGTAGGCAACTAAAACT	124
CLCA2	TTCCACCTCCTCCCACATTC GGGCTCTGATCTCTCCTTTGC	201
CLDN1	CCGTTGGCATGAAGTGTATG CCAGTGAAGAGAGCCTGACC	208
FGFR3	GAATTCAGTTGTTCGTTCTGTACTG AAAGTTCGTCGCTGGGTTAACA	172
FZD6	TGGCCTGAGGAGCTTGAATG GAGGCGCACACTGGTCAATT	202
RALA	TGCGGAATTCAAGTTACCAAT GGGTAGTGGCATCAGCCTAAGA	201
CD200	CCTGGTAATTCTTCTCGTCCTAATCT CTTTCGCTCCCACCTCTTTTG	200
CCND1	CCCTGACGGCCGAGAAG GGTCTGCGCGTGTTTGC	200
CCNE1	CTGGATGTTGACTGCCTTGAATT GCGACGCCCCTGAAGTG	200

2.4.4 Semi-quantitative PCR (semi-qPCR)

Semi-qPCR was performed using GeneAmp PCR System (Applied Biosystems, USA) in a 50µl reaction: 33.75µl water, 5µl 10x PCR Buffer II, 3µl 25mM $MgCl_2$, 1µl 10mM dNTPs, 1µl each of 10µM forward and reverse gene-specific primers, 0.25µl AmpliTaq Gold and 5µl of Human MTC™ Panel I or II (Clontech, USA) as the cDNA template. The reaction condition was as follows: 5min of 94°C, followed by 25, 30 or 35 cycles of 30sec each at 94°C, 59°C and 72°C, and finally 7min at 72°C. 5µl aliquots of the PCR products were visualized in 2% agarose gel.

2.4.5 Cloning of FJX1

The coding region of FJX1 was amplified from genomic DNA of NP460 cells using GC-RICH PCR System (Roche, Germany). The primers used were forward 5'-CCC*GGATCC*ACCATGGGCAGGAGGATG-3' and reverse 5'-G*GAATTC*CGAGTCCCAGACCGGCGGCCGTA-3' (italics signifies *BamHI* and *EcoRI* restriction sites in forward and reverse primers). The PCR product was electrophoresed on an agarose gel and a band at 1.3kb was excised from the agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen, the Netherlands). The purified PCR product was ligated into a cloning vector pGEM-T Easy (Promega, USA) in a 10µl ligation reaction: 5µl of 2X Rapid Ligation Buffer, 1µl (50ng) pGEM-T Easy Vector, 1µl of T4 DNA Ligase and 3µl of the PCR product. The ligation mixture was incubated overnight at 4°C prior to transformation into TOP10 competent *E.coli* cells. The competent cell/DNA mixture was incubated on ice for 20min, heat-shocked at 42°C for 30sec, and promptly placed on ice for 2min. 950µl of LB broth was added and the cells were shaken at 150 rpm, 37°C for 2hrs. 100µl of the transformation mix was spread on LB agar plate containing 100µg/ml ampicillin

(Sigma-Aldrich, USA) plus 0.5mM IPTG and 80µg/ml X-Gal. The plate was incubated overnight at 37°C. A single white colony bearing the plasmid with the gene insert was inoculated into 8mL of LB broth containing 100µg/mL ampicillin and cultured at 150 rpm, 37°C overnight. The plasmid was purified using MiniPrep Plasmid Extraction Kit (Qiagen, the Netherlands) following manufacturer's recommendation and sent for sequencing (FirstBase, Malaysia).

The FJX1 coding region was subsequently subcloned into a mammalian expression vector pcDNA3.1-V5/His-B (Invitrogen, USA) to generate a recombinant FJX1 protein tagged with V5 epitope at the C-terminal. 2µg of pcDNA3.1-V5/His-B plasmid or the purified pGEM-T Easy vector carrying FJX1 gene was digested using BamH1 and EcoR1 restriction enzymes: 3µl of 10X buffer, 0.3µl acetylated BSA, 0.75µl each of BamH1 and EcoR1 restriction enzymes in a 30µl reaction. The reaction was performed at 37°C for 3hr, before inactivation at 65°C for 15min. The digested DNA was separated in agarose gel and the DNA band of the correct molecular weight was excised from the agarose gel. The DNA content from the agarose was purified using QIAQuick Gel Extraction Kit (Qiagen, the Netherlands). The ligation reaction was carried out in a reaction containing 1µl T4 DNA Ligase (New England Biolabs, USA), 2µl 10X Buffer, 2µl pcDNA vector, and 15µl FJX1 DNA fragment. The reaction was performed for 10min at room temperature prior to transformation into competent *E.coli* cells as previously described.

A bacteria clone carrying the correct sequence was identified and the plasmid was purified using MaxiPrep Plasmid Extraction Kit (Qiagen, the Netherlands) following manufacturer's recommendation.

2.5 BIOCHEMISTRY

2.5.1 Protein Extraction

Cells at about 80% in confluency were washed twice with ice-cold PBS and incubated on ice for 20min in 1 or 2mL RIPA buffer [50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, 1% Halt-protease cocktail inhibitor (Roche, Germany), and 1mM Na_3VO_4]. The lysate was collected using a cell scraper and cleared via centrifugation at *10,000 x g* for 30min at 4°C. The supernatant was transferred to a new tube and stored in -80°C. An aliquot of the supernatant was used for protein concentration determination (Section 2.5.2).

2.5.2 Protein Concentration Determination

Protein concentration was determined using Coomassie Plus reagent (Pierce, Thermo Scientific). 300µl of Coomasie Plus reagent was incubated with 10µl of protein samples in a 96-well plate for 15min at room temperature. Absorbance at 595nm was measured in MultiSkan FC microplate reader (ThermoFischer Scientific, USA). The experiment was done triplicate per sample, and 10µl RIPA buffer plus 300µl Coomasie Plus was used as a blank reading. A standard curve was made using BSA solution in the range of 0 to 2,000µg/ml.

2.5.3 Western Blot

40µg protein samples were mixed with 6x sample buffer (6% SDS, 30% glycerin, 320mM Tris-HCl pH 6.8, 3% β-mercaptoethanol and trace bromophenol blue) and boiled for 5min at 95°C. The samples were then electrophoresed in SDS-PAGE, together with 4µl Precision Plus Protein™ Standards (Bio-Rad, USA).

The separated proteins were transferred to a nitrocellulose membrane with 0.45µm pore size (Thermo Scientific, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad, USA). The membrane was then blocked in blocking solution [5% non-fat skim milk in PBS] for 1hr before incubating with primary antibodies diluted in blocking solution for overnight at 4°C. The membrane was washed three times for 5min each with PBST [0.1% Tween-20 (Sigma-Aldrich, USA) in PBS]. The membrane was subsequently incubated with secondary antibodies diluted in blocking solution for 1hr at room temperature. After further washes with PBST (3x5min), the membrane was scanned using Odyssey Infrared Imaging System (Licor, USA). To reuse the blot with different primary antibodies, the affinity-bound antibodies were removed by incubating the membrane in 0.2M NaOH solution for 5min, followed by two washes in PBST. The membrane was subsequently blocked with the blocking solution, incubated with primary and secondary antibodies as described above. The antibodies and the concentration used are listed in Table 2.4.

2.5.4 Immunohistochemistry

Immunohistochemistry was performed using Dako REAL™ EnVision Detection System (DakoCytomation, Denmark). 5µm sections of formalin-fixed paraffin-embedded tissues were deparafffinized in 2 xylene soaks for 5min each and rehydrated through a series of graded ethanol (2 times in 100%, 2 times in 95%, and once in 70% ethanol) for 3min each. The tissue sections were treated with Antigen Retrieval Solution High pH (Link Technology, USA) pH 6.0 for 10 minutes in a microwave for antigen retrieval. After cooling to room temperature and two PBS washes for 5min each, sections were incubated in Dako REAL™ Peroxidase-Blocking Solution for 15min. The sections were subsequently rinsed twice with PBS and incubated with anti-human FJX1 Rabbit Polyclonal antibody (#ARP47013, Aviva

Systems Biology, USA) at 1:500 dilution in PBS. After 1hr incubation, the sections were rinsed with PBS and incubated with Dako REAL™ Envision™/HRP, Rabbit/Mouse (ENV) peroxidase-conjugated polymer for 30min. After further washes in PBS, the sections were developed using diaminobenzidine (DAB) chromogen and the reaction was stopped by subjecting the slides in running water. The sections were counterstained with Harris' haematoxylin (BDH Laboratories, USA), followed by water rinse, dehydration in graded alcohols, cleared in xylene and mounted in DPX (Fluka Biochemika, Switzerland).

2.5.5 Immunocytochemistry

Immunostaining of cultured cells was performed using Dako REAL™ EnVision Detection System (DakoCytomation, Denmark). 5hr after transfection, cells were trypsinized from a 6-well plate and seeded on a sterile glass slide placed in a 100mm^2 culture dish. The cells were allowed to adhere to the glass slide for at least two hours before adding 10ml RPMI media into the culture dish. After incubation overnight at 37°C, the glass slide was rinsed twice with PBS and fixed in acetone for 10min. After two washes in PBS, primary antibodies diluted in PBS were applied for 30min at room temperature. The slides were later rinsed with PBST 0.1% and incubated with labeled polymer conjugate for 30min at room temperature. Subsequently, the slides were rinsed with PBST 0.1%. The slides were developed in DAB chromogen for 10min and washed with water. Counterstaining for nuclei was done using Harris' haematoxylin (BDH Laboratories, USA). For mounting steps, the slides were initially immersed twice in ethanol for 2min each, following a single immersion in xylene for 3min. Mounting on a coverslip was done using DPX (Fluka Biochemika, USA). Antibodies and their concentrations are listed in Table 2.4.

Table 2.4: Antibodies Used in Western Blotting and Immunohistochemistry

Antibody	Concentration in Western	Concentration in immunohistochemistry
Mouse anti-Cyclin D1 (M7155, DakoCytomation, Denmark)	1:50	*Not done*
Mouse anti-Cytokeratin 5 (NCL-L-CK5, Leica Microsystems, Germany)	1:200	1:50
Mouse anti-Cytokeratin 10 (M7002, DakoCytomation, Denmark)	*Not suitable for Western*	1:50
Mouse anti-Vimentin (M0725, DakoCytomation, Denmark)	1:100	1:25
Mouse anti-β-Catenin (M35339, DakoCytomation, Denmark)	1:200	1:25
Rabbit anti-Twist (sc-15393, Santa Cruz, USA)	1:200	*Not done*
Rabbit anti-Snail (ab85931, Abcam,USA)	1:1,000	*Not done*
Rabbit anti-ZO1 (ab59720, Abcam, USA)	1:200	*Not done*
Rabbit anti-E-cadherin (abEP700Y, Abcam, USA)	1:2,000	1:50
Mouse anti-alpha tubulin (#T5168, Sigma, USA)	1:2,000	*Not applicable*
Rabbit anti-V5 (#ab9116, Abcam, USA)	1:2,000	1:500

Table 2.4, continued

Antibody	Concentration in Western	Concentration in immunohistochemistry
Goat anti-Mouse IRDye® 680 (#926-32220, Odyssey®, USA)	1:5,000	*Not applicable*
Goat anti-Rabbit IRDye® 800 (#926-32211, Odyssey®, USA)	1:5,000	*Not applicable*

2.6 *IN VITRO* ASSAYS

2.6.1 Transient Overexpression of Recombinant FJX:V5 Protein

$2.5x10^4$ SUNE1 or $3.5x10^4$ TWO4 cells per well were seeded in 6-well plates and cultured overnight to reach 80-90% confluency. 250µl serum free media were mixed with 4µg plasmid DNA in an Eppendorf tube. In another tube 250µl serum free media was mixed with 16µg Lipofectamine™ 2000 Transfection Reagent (Invitrogen, USA). The contents of the two tubes were incubated separately for 5min and then combined. After incubation for another 20min, the transfection mixture was added to the cells in each well containing 2mL complete RPMI.

2.6.2 Transient Knockdown of Endogenous FJX1 Transcript

$7.5x10^4$ HK1 cells were seeded in a well of 6-well plates to achieve 80-90% confluency after overnight incubation. The cells were transfected with either ON-TARGETplus SMARTpool siRNA against FJX1 or ON-TARGETplus Non-Targeting Pool siRNA (Dharmacon, Thermo Scientific, USA). Briefly, 200µl serum free media containing 5µl of 20µM siRNA and another 200µl serum free media containing 5µl DharmaFECT 1 were incubated separately in Eppendorf tubes for 5min. Subsequently, the contents from the tubes were combined and incubated for another 20min. This 400µl transfection mix was later added to the cells in a single well of 6-well plate containing 1.6mL complete RPMI. Sequences of the FJX1-targeting siRNA oligonucleotide species in the pool are: 5'-CGGAGCAGAUUCAGGGCGA-3', 5'-AGUACAAUGGACCGACUUA-3', 5'-UCGACUACCUGACGGCCA-3', and 5'-GGACUUAGUGUCACCGGGA-3'.

2.6.3 Proliferation Assay

24hr post-transfection with plasmid construct or siRNA, 50,000 TWO4 or HK1 cells per well were seeded into 6-well plates. Cells in triplicate wells were trypsinized using Trypsin-EDTA and counted using CASY® cell counter every day. The media was changed on day 3.

The mean cell counts and standard errors were calculated and two tailed Student's *t*-test was used to determine the statistical significance of the differences. $P<0.05$ was taken as being statistically significant.

2.6.4 Transwell Migration Assay

Cell migration assay were performed using transwell inserts (8µm pore size; BD Biosciences, USA) in 24 Well MultiWell™ Plate (BD Biosciences, USA). 1 day post-transfection with either plasmid or siRNA, the cells were treated with 15µl/mL mitomycin C for 2hr to limit their proliferative potential. The mitomycin C-treated cells were washed twice with migration buffer (0.1% BSA in serum-free RPMI), and 50,000 cells in 100µl of migration buffer were seeded into the inserts. The inserts were placed into the wells that contain 450µl migration buffer plus 50µl FBS. The plate was incubated in tissue culture incubator to allow cells to migrate. After 24hr, 500µl trypsin-EDTA was used to detach the migrated cells from the bottom of the membrane for at least 20min, and the total migrated cells were counted using the CASY® cell counter. The experiments were done in triplicates.

The mean migrated cell counts and standard errors were calculated and two tailed Student's *t*-test was used to determine the statistical significance. $P<0.05$ was taken as being statistically significant.

2.6.5 Transwell Invasion Assay

Cell invasion assay were performed using transwell inserts (8µm pore size; BD Biosciences, USA) in 24 Well MultiWell™ Plate (BD Biosciences, USA). 1 day post-transfection with either plasmid or siRNAs, the cells were treated with 15µl/mL mitomycin C for two hours to limit their proliferative potential. The inside of transwell inserts was coated with 70µl of 2:1 mix of serum-free RPMI and Matrigel (BD Biosciences, USA) for one hour. The mitomycin C-treated cells were washed twice with migration buffer (0.1% BSA in serum-free RPMI), and 50,000 cells in 200µl migration buffer were seeded on top of the matrigel layer in the insert. 500µl 10% RPMI was placed in the wells and the plate was incubated in the tissue culture incubator. After 48hr, 500µl trypsin-EDTA was used to detach the cells from the bottom of the insert and the total invaded cells were counted using CASY® cell counter. The experiments were done in triplicates.

The mean invaded cell counts and standard errors were calculated and two tailed Student's *t*-test was used to determine the statistical significance of the differences in cell numbers between two different treatments. $P<0.05$ was taken as being statistically significant.

2.6.6 Soft-agar Assay

Low-melting agarose VII (Sigma-Aldrich, USA) in a Schott bottle was sterilized using UV rays. Complete RPMI was added to make a 1% agarose solution, and this solution was microwaved at low power. To prepare the 1% bottom agar layer, 2mL of the 1% agarose/RPMI was placed in a well of 6-well plates and left for at least 1 hour to solidify. The 0.5% top agar layer was prepared by mixing equal volumes of a suspension of 2,000cells/mL in complete RPMI and the 1% agarose/RPMI solution. 2mL of the cell/agarose solution was quickly dispensed on top of the bottom layer and, left for another hour to solidify, before

being put in the 37°C incubator. For assays using 12-well plates, 1,000 cells in 1ml top agar were added to 1ml of the bottom agar. Total number of colonies was counted under the microscope after 3 weeks.

The mean colony counts and standard errors were calculated and two tailed Student's *t*-test was used to determine the statistical significance of the differences in cell numbers between two different treatments. $P<0.05$ was taken as being statistically significant.