Comparative expression analysis of the ribosomal protein gene, RPS15 between normal and tumor cell lines of nasopharyngeal carcinoma

Cassandra Chee Sheau Mei (35640)

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Cassandra Chee Sheau Mei (35640)

A Thesis Submitted in Partial Fulfillment of the Requirement of

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Supervisor: AP Dr. Edmund Sim Ui Hang

Resource Biotechnology

Department of Molecular Biology

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List of Abbreviations

AGE	Agarose Gel Electrophoresis
AP	Activator Protein
AP-1	Activator Protein 1
EBV	Epstein-Barr virus Infection
EBNA	Epstein-Barr virus Nuclear Antigen
EtBr	Ethidium Bromide
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
LMP1	Latent Membrane Protein 1
NCBI	National Center for Biotechnology Information
NKC	Non-keratinizing Carcinoma
NPC	Nasopharyngeal Carcinoma
NPE	Nasopharyngeal Epithelium
NRF	Nuclear Respiratory Factor
RNA	Ribonucleic Acid
RPS15	Ribosomal Protein Small 15
RT-PCR	Reverse Transcription Polymerase Reaction
SCC	Squamous Cell Carcinoma
Tm	Melting Temperature
UC	Undifferentiated Carcinoma
VCA	Viral Capsid Antigen
WHO	World Health Organization

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Resource Biotechnology
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ABSTRACT

Cancer is associated in a complex alteration in gene expression patterns in the ribosomal proteins that include many different ribosomal genes. Ribosomal proteins have extraribosomal functions and can be involved in other variety of activities other than protein synthesis. The differences in the gene expression of an individual ribosomal protein can affect a specific cell and cause a transformation in the cell, which could result in cell death or proliferation. The *RPS15* gene is up regulated in poorly differentiated nasopharyngeal carcinoma (NPC). Our research aim is to detect different expression patterns in NPC cell lines and normal human nasopharyngeal cell line with the incorporation of Reverse Transcriptase PCR (RT-PCR). This study reveals specific PCR products with the estimated size of 115bp for *RPS15.* The *RPS15* gene in NPC cell line was found to be up regulated in comparison to normal human nasopharyngeal cell lines.

Key word: gene expression patterns, ribosomal proteins, nasopharyngeal carcinoma, *RPS15*, RT-PCR

ABSTRAK

Kanser dikaitkan dengan perubahan kompleks dalam corak ekspresi gen dalam protein ribosom yang termasuk banyak gen ribosom berbeza. Protein ribosom mempunyai fungsi "extraribosomal" dan boleh terlibat dalam pelbagai kegiatan-kegiatan selain daripada sintesis protein. Perbezaan dalam ungkapan gen daripada protein ribosom individu boleh menjejaskan sel tertentu dan menyebabkan perubahan dalam sel, yang boleh menyebabkan kematian atau proliferasi sel. Gen RPS15 adalah dikatakan mempunyai eksperi gene yang lebih di sel karsinoma nasofarinks (NPC) berbanding sel normal. Tujuan penyelidikan kami adalah untuk berbandingkan ekspresi gen RPS15 dalam sel NPC dan sel normal nasofarinks dengan menggunakan teknik "Reverse transcriptase PCR" (RT-PCR). Kajian ini menghasilkan produk PCR dengan anggaran saiz 115bp untuk RPS15. Gen RPS15 dalam sel NPC tidak mununjukkan perbezaan dalam ekspresi gen berbanding dengan sel normal nasofarinks.

Kata Kunci: gen corak expresi, protein ribosom, karsinoma nasofarinks, RPS15, RT-PCR

1.0 Introduction

Nasopharyngeal cancer is a cancer that starts at the nasopharyngeal, which is the upper part of the throat behind the nose, near the base of the skull. Nasopharyngeal carcinoma (NPC) is a malignant tumor that begins in the epithelial nasopharyngeal cells. Poorly differentiated squamous cell carcinoma (SCC) cell line is common in NPC patients in South China and has a high level on NPC incidence and mortality reported, according to Wei *et al.* (2014). Ribosomal proteins are partly responsible for NPC incidence apart from Epstein-Barr virus infection (EBV) and other causes example diet, environment, gender, age, race, and many others.

Research has shown that ribosomal proteins play a role in NPC cases, as there are differential gene expression when compared between tumor and normal cell lines. Ribosomal proteins are proteins that makes up of ribosomal subunits that are involve in the cellular process of translation. According to de Las Heras-Rubio *et al*. (2014), ribosomal proteins link with extraribosomal functions are associated with the proliferation in the cells. The expression patterns of ribosomal proteins seem to be deregulated in some human because of genetic mutation and appears to be involve in the tumorigenic process and its progression and metastasis (de Las Heras-Rubio *et al*., 2014). Alteration or mutation in the protein sequences causes the up-regulation or down-regulation of certain protein and will affect the protein translation, thus changing the synthesis of proteins that plays a major role in the beginning of cancer (Montanaro *et al*., 2008). Changes in tumor suppressors or proto-oncogenes either affect the conformation of the mature ribosome or control the activity of proteins, which is known as translation factor and also is responsible for the uncontrollable proliferation of the cell and up-regulation of ribosomes biogenesis (Ruggero and Pandolfi, 2003; Montanaro *et al*. 2008). Any disturbance in the regulation of the protein biosynthesis has been proven to lead to the changes in the cell cycle and regulation of cell growth (Ruggero and Pandolfi, 2003).

Studies suggested that ribosomal protein genes to be a candidate cancer-causing genes when tested using a Zebrafish as a model (Ma *et al.*, 2012). A differential expression pattern in ribosomal proteins could lead to the formation of tumors, as shown

by Naora and Naora (1999) when *RPS3a* expression was enhanced causing the formation of tumors in nude mice. Ribosomal protein genes, such as *RPS15* regulates Mdm2-p53-Mdmx network that inhibits the p53, which is a tumor suppressor in cells, from allowing the cell to undergo cell arrest or apoptosis therefore allowing the proliferation of the cells (Daftuar, 2013). This gene is a protein-coding gene and is situated in the cytoplasm. It sequence proteins that belongs to the S19P family of the ribosomal proteins at chromosome 19p13.3. It is important and required in the final cytoplasmic maturation and assembly of the 40S subunit (Robledo *et al*., 2008). The halt on the expression of *RPS15* cause the decrease and eventually the disappearance of the free 40S subunit pool and an increase of the free 60S subunit (Rouquette *et al*., 2005). Studies have shown that *RPS15* has proved to be vital in the biological processes in humans but also can be highly active in cancerous and tumor cells.

Findings by Fang *et al.* (2008) revealed an up regulation of *RPS15* in the NPC primary tumor, however their findings were focused on poorly differentiated squamous cell carcinoma (SCC). We mainly focused on the expression pattern in differentiated SCC using HK1 cell line. Therefore, a study was conducted using *RPS15* gene taken from both normal nasopharyngeal cell line and NPC cell line, which are NP69 and HK1 respectively and extracted using TRIzol method, followed by quantification and quality check of the extracted RNA, RT-PCR, cloning and lastly agarose gel electrophoresis (AGE) and data analysis.

The aim for this study was to compare the expression patterns of *RPS15* in normal nasopharyngeal cell line, NP69 and NPC cell line, HK1.

The objectives for this study were:

1) To observe and detect *RPS15* expression in NPC cell line, HK1
2) To observe the difference in expression pattern of *RPS15* in both NPC cell line, HK1 and normal nasopharyngeal cell line, NP69

The study revealed differential expression patterns of *RPS15* gene in normal NP cell line and NPC cell line based on the estimated sizes of PCR products of 115bp. The hypothesis of this study is based on the relative quantification and statistical result using independent t-test showing that *RPS15* is over-expressed in NPC cell line compared to normal nasopharyngeal cell line.

2.0 Literature Review

2.1 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is a cancer that happens in the nasopharynx, in the upper part of the throat behind the nose and near the base of the skull (Figure 1).

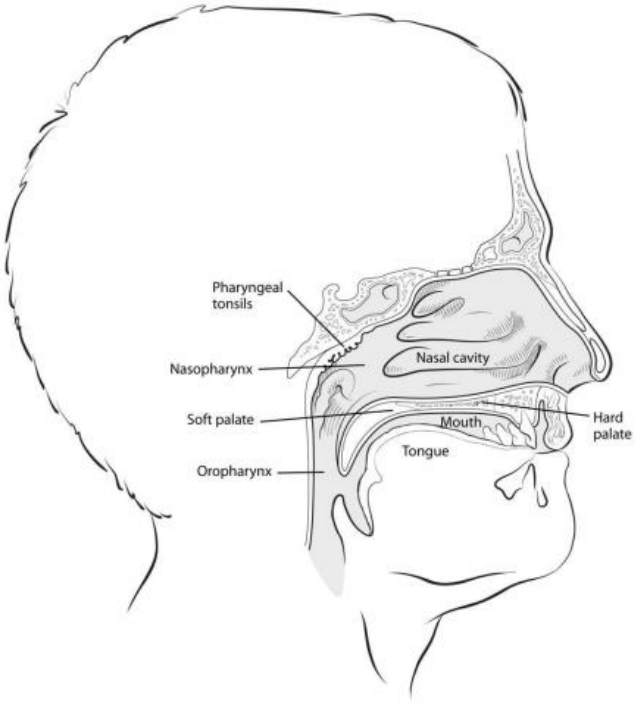

Figure 1: Anatomy of the pharynx (American Cancer Society, 2013)

Tumor that forms in the nasopharynx that is malignant tumor and not benign are harmful and commonly starts in the nasopharyngeal epithelial cells. NPC has been classified by the World Health Organization (WHO) into three types which are squamous cell carcinoma (SCC, WHO type 1), non-keratinizing differentiated carcinoma (NKC, WHO type 2) and undifferentiated carcinoma (UC, WHO type 3) and possesses different characteristics of the NPC (Pathmanathan *et al*., 1995) (Figure 2).

(a) (b) (c)

Figure 2: Microscope view of SCC (a), NKC (b) and UC (c) (PathologyOutlines.com, 2014)

Type 1, SCC has intercellular bridges and the keratin is either intracellular or extra-cellular (Pathmanathan *et al*., 1995). The nasopharyngeal origin of SCC can be well, moderately, or poorly differentiated cancers (Pathmanathan *et al*., 1995). Type 2, NKC and Type 3, UC are easily recognized from SCC by the lack of squamous differentiation and a significant lymphoid infiltration (Pathmanathan *et al*., 1995). Both of these NPC types are related with Epstein-Barr virus infection (EBV) (Pathmanathan *et al*., 1995). The type 2, NKC shows a pavemented or layered arrangement and are clearly separated by cell margins (Pathmanathan *et al*., 1995). The type 3, UC however, has unclear cell borders, looks like syncytia in sheet-like masses and has prominent nucleoli (Pathmanathan *et al*., 1995). There are several factors that can cause a person to have NPC like gender, race, diet, EBV, genetic factors, tobacco and environment. Unfortunately the exact cause of NPC is still unknown, however scientist are studying on how EBV is involve in the development of NPC and how ribosomal proteins are related to its becoming.

2.2 Cell Line

Cancer cell cultures are grown in Petri dishes that have been the groundwork of cancer research, cancer biology and drug treatments (Borrell, 2010). However, researches questioned whether cell lines are as compatible as primary tumors. Primary tumors are

more accurate as it is directly from the source itself. The major problem of cell lines is that not all cancers are able to grow indefinitely in laboratories (Borrell, 2010). Cell lines resemble primary tumors but there could be a slight mutation in the cell lines as it is grown in an artificial environment. The benefits of using cell lines is that they do not contain non-cancerous cells that are found in primary tumors therefore perfect for finding deletions in the cancer genome (Borrell, 2010). Sequencing cancer cell lines is ideal for short-term study of cancer genomes however primary tumors will be more reliable overtime as the number of patients diagnose with cancer are increasing (Borrell, 2010).

2.2.1 HK1

Based on the findings by Huang *et al.* (1980), HK1, a nasopharyngeal carcinoma (NPC) cell line, has shown to be well-differentiated squamous cell carcinoma (SCC). However, well-differentiated SCC is rare in NPC patients. Undifferentiated carcinoma (UC) is more commonly observed in NPC patients especially in the Southern China. The well-differentiated SCC derived from NPC cell line shows no detectable presence of Epstein-Barr virus nuclear antigen (EBNA) and viral capsid antigen (VCA) when Epstein-Barr virus (EBV) was exposed to the cells (Huang *et al.*, 1980). This demonstrates the absence of EBV in HK1, therefore concluding that HK1 is an EBV-negative cell line. Chromosomal studies on HK1 show a high addition and deletion in the number of chromosomes (Huang *et al.*, 1980).

2.2.2 NP69

NP69 is an immortal nasopharyngeal epithelial cell line and has been established from primary nonmalignant nasopharyngeal epithelial cells and serves as a standard premalignant nasopharyngeal epithelial (NPE) and a normal control for nasopharyngeal carcinoma (NPC) cell line. According to findings by Lo *et al.* (2003), EBV-encoded latent membrane protein 1 (LMP1) causes many phenotypic alterations in NP69 cells which includes, morphological transformation, increase in cell proliferation, anchorage-independent growth, resistance to serum free-induced cell death and enhanced cell migration and invasion. LMP1 causes an up regulation of integrin α6 that may have assisted in tumor metastasis (Lo *et al.*, 2001). NP69, based on the research done by Lo *et*

al. (2003), changes from a typical epithelial cobblestone to an elongated and fibroblastoid shape when induced with LMP1. Figure 3 shows the experiment conducted by Lo *et al.* (2003) proving that LMP1 changes the morphological shape of the cells and also increase cell proliferation.

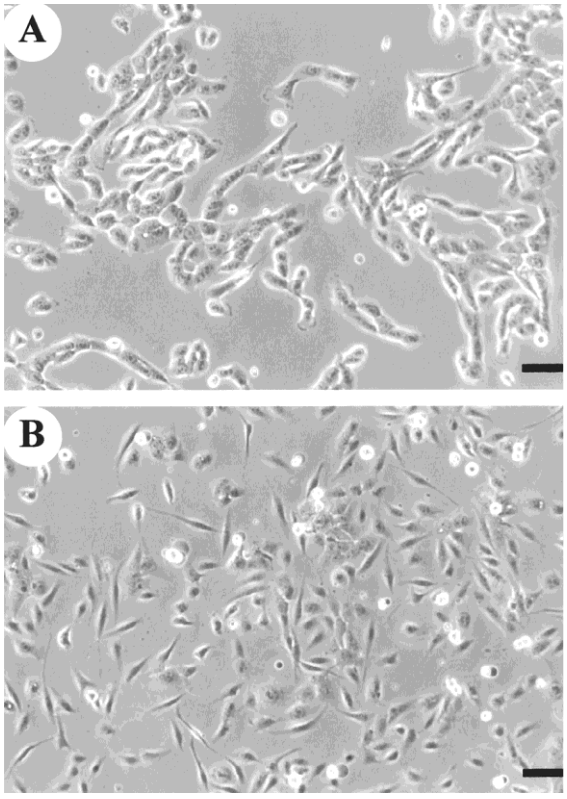

Figure 3: Morphologies of NP69 nasopharyngeal epithelial cells. (A), NP69 control cells; (B), LMP1-expressing NP69 cells (Lo *et al*., 2003)

2.3 Ribosomal Proteins and *RPS15*

Genes in the RPS family gives instructions for producing ribosomal proteins that makes up part of the cellular structure called ribosomes. Ribosomes catalyze protein synthesis. Ribosomes consist of 2 subunits, which are the small (40s) and large (60s) subunits. Each ribosomal protein is part of a huge ribonucleoprotein (Ishii *et al.*, 2006). Ribosomal subunit accumulation needs a deposition of many ribosomal proteins on ribosomal RNA (rRNA) to form two ribonucleoprotein particles, a small and a large subunit that assist in protein translation (Wild *et al*., 2010). RPS15 has been found to be located in the 40S subunit based on the findings by Kitagawa *et al.* (1991).

Ribosomal proteins genes have a unified control mechanism for transcription and translation and are able to manage its own synthesis at the translational level and are involve in the translational regulation (Ishii *et al.*, 2006; Stelzl *et al*., 2001). Ribosomal proteins are individual proteins and are separated by their gene expression level and tissue-specificity, as they are control by different regulators, optimal amino acids and codon. Therefore, they are able to perform extra-ribosomal functions as independent polypeptides (Ishii *et al.*, 2006). According to the findings by Ishii *et al.* (2006), *RPS15* has a thought to have a nuclear respiratory factor (NRF) and activator protein (AP) transcription bound, where AP-1 transcriptional factor is said to be involved in the regulation of proliferation, differentiation, apoptosis and transformation and NRF involve in transcription and act as a transcriptional activator. Ribosome assembly and function is reliant on the stoichiometric stability of ribosomal proteins, whereby a fall in the levels of one or more ribosomal proteins may possibly decrease the efficiency of, or even reduce in operational, the translational apparatus, and thus signal the cell to self destruct (Naora and Naora, 1999). The involvement of ribosomal protein, RPS3a in the cell transformation and apoptosis reveals that individual ribosomal proteins and changes in their expression involves in and regulates an extensive variety of activities correlated with cell growth and death (Naora and Naora, 1999). However, the change in expression of an individual ribosomal protein gene can vary depending on the type and state of the cell and also the presence of other signals (Naora and Naora, 1999). A down-regulation or up-regulation of an individual ribosomal protein gene does not necessarily lead to cell death. According to Naora and

Naora (1999), the expression level of a ribosomal component that is close to the required threshold in a particular cell type may reduce and that the cell that is more sensitive to the reduction in the expression will produce a phenotype that is cell-type specific.

This gene belongs to the S19 protein family located and expressed at the chromosome 19p13.3 (Figure 4). *RPS15* is similar to the S19 protein in bacteria and archeons, and is situated in the head of the 40S subunit and is suggested to be involved in the initiation and elongation steps of translation (Rouquette *et al*., 2005; Kitagawa *et al*., 1991). According to Ishii *et al.* (2006), *RPS15* were expressed in skin, fetal brain and spleen. *RPS15* has been observed in many tumor cells and is highly expressed such as insulinomas, esophageal and colon cancers and also during the differentiation phase of liver regeneration (Kitagawa *et* al., 1991).

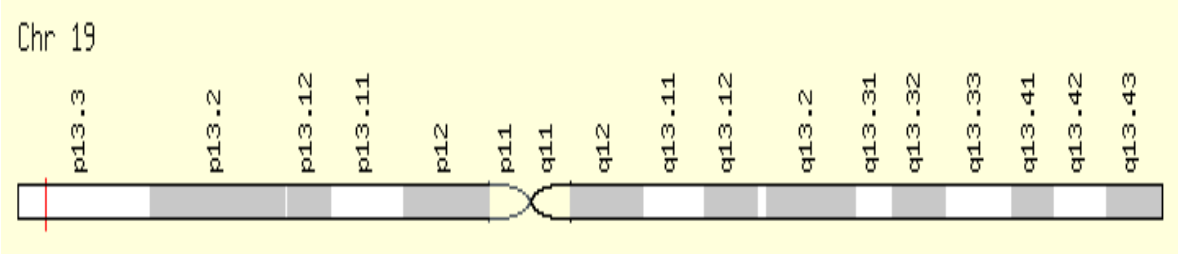

Figure 4: Location of *RPS15* in S19 chromosome (Genecards, n.d.)

Findings by Marion and Marion (1988), reveals that the deterioration of ribosomal proteins S2, S6, S10, S14, S15 and S25 can cause the segmentary orientation of 40S subunits. Figure 5 shows the two-dimensional electrophoresis of proteins extracted from

40S subunit that has been treated with immobilized trypsin. This verifies the presence of a large region of S15 that is situated abundantly at the surface of the 40S subunit.

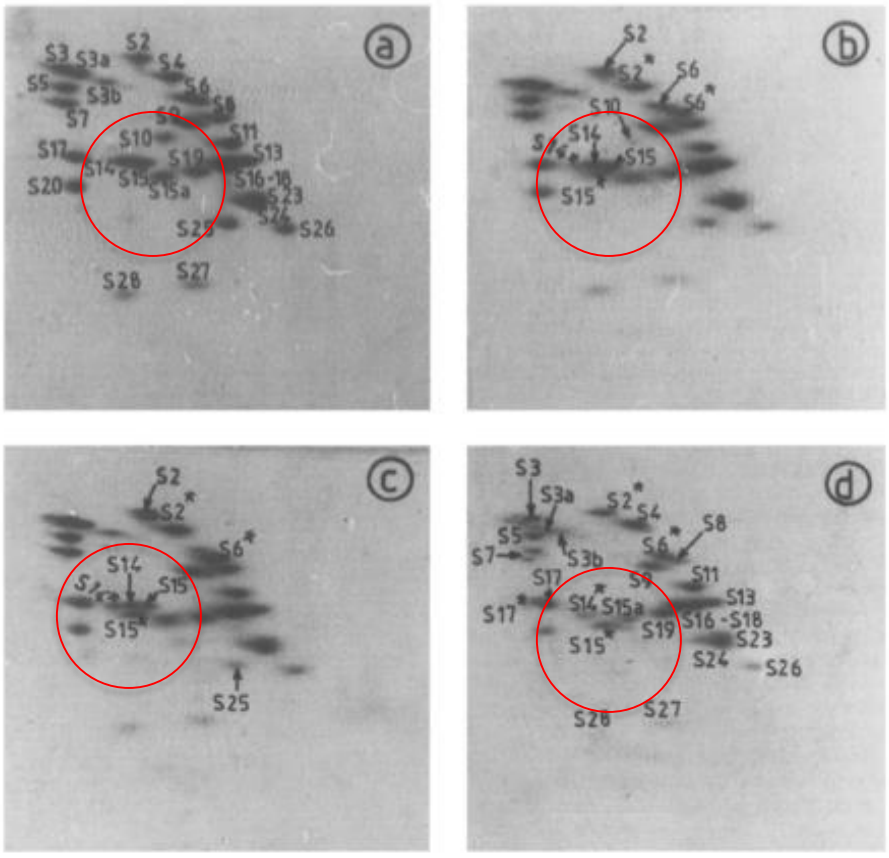

Figure 5: Two-dimensional electrophoresis of proteins extracted from 40S subunit; (a) shows the initial incubation, (b) 70 minutes after incubation, (c) 90 minutes after incubation and lastly (d) 300 minutes after incubation. (Marion and Marion, 1988).

Therefore, *RPS15* gene is important and required in the final cytoplasmic maturation and assembly of the 40S subunit as the reduction of *RPS15* in human cell lines causes the decrease in 40S subunits and 80S mature ribosomes (Robledo *et al*., 2008). As cited in Rouquette *et al*. (2005), *RPS15* is needed for the nuclear export of the pre-40S particles. It

may be directly or indirectly communicate with the nuclear transport machinery (Leger-Silvestre *et al*., 2004). *RPS15* is required for nuclear transport of the pre-40S particles (Rouquette *et al.,* 2005). The halt on the expression of RPS15 cause the decrease and eventually vanishing of the free 40S subunit pool and an increase the free 60S subunit (Rouquette *et al*., 2005). Disappearance of *RPS15* intervenes with 40S biogenesis in human cells and leads to nuclear 40S biogenesis defect (Wild *et al.,* 2010).

As stated by Naora and Naora (1999), the ribosome protein expression increases in actively proliferating cells and that the increased in ribosome biogenesis is a common feature of growth, whether it be normal or cancerous. The study by Naora and Naora (1999) shows that the increase in RPS3a expression is able to induce transformation and is involve in the formation of tumors in nude mice. However not all of the ribosomal protein genes whose expression is allegedly up-regulated plays a direct role in oncogenesis but there are significant evidences that some translation initiation factors can be involved in the oncogenic transformation (Naora and Naora, 1999).

3.0 Materials and Methods

The materials and reagents and also tools and apparatus used in this study are as shown in Table 1 and 2 respectively.

Table 1: Materials and Reagents

Materials and Reagents	Brand	Location
dNTP Mix	Promega	UK
10x Taq Buffer with KCl	Fermentas	USA
25mM $MgCl_2$	Promega	UK
M-MLV RT	Promega	UK
Recombinant Taq DNA Polymerase	Fermentas	USA
TRIzol Reagent	Invitrogen™	USA
Gene Ruler DNA Ladder Mix	Fermentas	USA

Table 2: Tools and Apparatus

Tools and Apparatus	Brand	Location
E-Centrifuge	Hettich Zentrifugen	Germany
Express Cool Turbo Freezer (GR-T452ZV)	LG	Korea
NCBI primer designing tool	http://www.ncbi.nlm.nih.gov/	-
PCR machine	SensoQuest	Germany
Power Pack™ Power Supply 3000	Major Science	USA
RNA Wood Hood	Köttermann	Germany
TotalLab Quant Software	TotalLab	USA
UV Spectrophotometer	Ultrospec 1100 pro	UK
UV Transluminator	Wise UV.WUV-M10	Korea

3.1 Cell Culture

For this study, NP69 and HK1 were used as normal nasopharyngeal cell line and nasopharyngeal carcinoma (NPC) cell line, respectively. Table 3 shows the source and type of cell lines. The NP69 and HK1 cell lines were revived and immersed with DKSFM and RPMI media, respectively.

Table 3: Source of cell lines

Designated Name	Type of Cell Line	Obtained from
NP69	Normal Human Nasopharyngeal Epithelial	Prof. Tsao Sai Wah from Department of Anatomy, University of Hong Kong
HK1	Human Nasopharyngeal Carcinoma	Faculty of Health and Medical Science, Universiti Malaysia Sarawak

3.2 Primer Design

Forward and reverse primers for *RPS15* and *GAPDH* were designed using NCBI primer designing tool, whereby *GAPDH* (glyceraldehyde-3-phosphate dehydrogenesis) acts as the reference gene and used to normalize the band intensity of the cell lines. The mRNA sequences of both *RPS15* and *GAPDH* were retrieved from the NCBI database. *GAPDH* was aligned using alignment sequences nucleotide BLAST (NCBI, US). The primers were checked for hairpin formation using Oligocalc (DANA-Faber Cancer Institute) and also for secondary calculation using OligoAnalyzer (Integrated DNA Technologies, US). The sequences were searched in Blastn (G-blastn, Source Forge) to validate its similarities to our desired sequence only. Table 4 shows the list of primers.

Table 4: List of primers (NCBI, primer blast)

Primers	**Sequences (5'-3')**	**Length (bp)**	**GC Content (%)**	**Expected Product Length (bp)**
RPS15 (F)	GAA GAC GCA CCT GCG GGA CA	20	65.00	115
RPS15 (R)	GTGG CCGA TCAT CTCG GGCT TG	22	63.64	
GAPDH (F)	AAG ATC ATC AGC AAT GGC TC	20	45.00	509
GAPDH (R)	TAC CAG GAC ATG AGC TTG AC	20	50.00	

3.3 RNA Extraction

The RNA was extracted from normal nasopharyngeal cell line, NP69 and nasopharyngeal carcinoma (NPC) cell line, HK1 using TRIzol method (Chomczynski and Sacchi, 1987). The flask containing the cultured cell lines were taken and placed inside the RNA hood. The media inside the flask was removed and the cell monolayer was rinsed with 1 ml of PBS. 1 ml of TRIzol reagent (Invitrogen™, USA) per 3.5 cm diameter dish was added into the flask and using a cell scrapper, the cells were scraped off the flask, thoroughly and hard for 1 minute and then mechanically lysed by pipetting up and down several times and incubated for 5 minutes. The TRIzol mixture was transferred into a clean sterile tube and 0.2 ml of chloroform was added and vortexed for 15 seconds and then incubated for 5