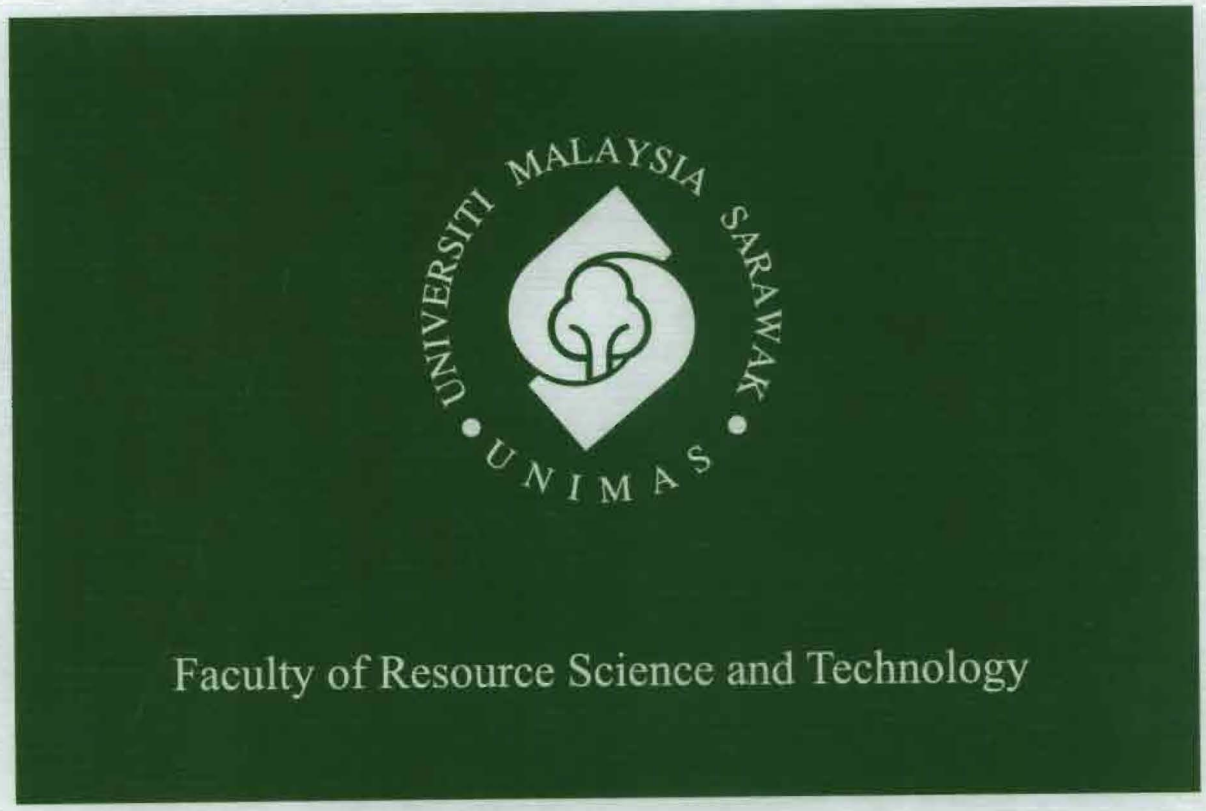

Comparison of *RPL14* Expression Levels between Normal and Tumour Cells of Human Nasopharyngeal Epithelium

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Bachelor of Science with Honours
(Resource Biotechnology)
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Table of Contents

Acknowledgement	I
Declaration	II-III
Table of Content	IV-V
List of Abbreviations	VI
List of Tables and Figures	VII-IX
Abstract	1
1.0 **Introduction**	2-4
2.0 **Literature Review**	5
2.1 Human Nasopharyngeal System	5
2.2 Nasopharyngeal Carcinoma (NPC)	5
2.2.1 Biology of NPC	5-6
2.2.2 Histology of NPC	6
2.2.3 Etiology of NPC	7
2.2.4 Human Nasopharyngeal Cell Line (HK1 & NP69)	8
2.2.4.1 HK1	8
2.2.4.2 NP69	8-9
2.3 Ribosomal Protein and *RPL14*	9
2.3.1 Ribosomal Protein	9-10
2.3.2 *RPL14*	10
2.4 Housekeeping gene (*GAPDH*)	11
2.4.1 *GAPDH*	11

3.0 **Materials and Method** 12

3.1 Cell Culture 13

3.2 Total RNA Extraction 13-14

3.3 RNA Quantification and Quality Check 14

3.4 Primer Synthesis 15-16

3.5 cDNA Synthesis 16

3.6 Polymerase Chain Reaction 17

3.7 Agarose Gel Electrophoresis and Sequencing 18

3.8 Data Analysis 18-19

4.0 **Results** 20

4.1 RNA Quantification 20-21

4.2 Polymerase Chain Reaction Optimization 21

4.2.1 Annealing Temperature Optimization 21-23

4.3 Polymerase Chain Reaction Profiling 23-24

4.4 Sequencing 25-29

5.0 **Data Analysis** 30-32

6.0 **Discussion** 33-35

7.0 **Conclusion and Recommendation** 36

8.0 **References** 37-39

9.0 **Appendix** 40-48

List of Abbreviations

EBV	=	Epstein-Barr Virus
AGE	=	Agarose Gel Electrophoresis
ASR	=	Age-Standardized Incidence Rate
EtBr	=	Ethidium Bromide
GADPH	=	Glyceraldehyde-3-Phosphate Dehydrogenase
IgA	=	Immunoglobulin A
L, 60S	=	Large, 60 Subunits
LMP	=	Latent Membrane Protein
NPC	=	Nasopharyngeal Carcinoma
PAR	=	Protein Associated with Ribosome
RNA	=	Ribonucleic acid
RP	=	Ribosomal Protein
RPL14	=	Ribosomal Protein L14
RPS	=	Ribosomal Protein Small Subunits
rRNA	=	ribosomal Ribonucleic Acid
UCNT	=	Undifferentiated Carcinoma of Nasopharyngeal Types
WHO	=	World Health Organization

List of Tables

Table		Page
Table 3.1	Materials and reagents	12
Table 3.2	Tools and apparatus	12
Table 3.3	Sources of cell lines	13
Table 3.4	Information of primers	16
Table 3.5	PCR master mix	17
Table 3.6	PCR thermocycling profiling	17
Table 4.1	Quantification of total RNA extraction from NP69 and HK1 using UV-Spectrophotometer	21
Table 4.2	Optimized PCR parameters for gene expression of gene interest in NP69 and HK1 cell lines	24
Table 4.3	Expected product length of the genes	24
Table 5.1	The band intensity of PCR products of *RPL14* and *GAPDH* in both cell lines, HK1 and NP69	30
Table 5.2	Normalized values and fold difference values of *RPL14* in NP69 and HK1 cell lines	32
Table 5.3	Statistical significance of the gene expression for *RPL14* in NPC cell lined compared to normal nasopharyngeal cell line by Unpaired student's t-test	32

List of Figures

Figure		Page
Figure 2.1	Location of nasopharyngeal tumour	6
Figure 2.2	The location of *RPL14* in chromosome 3	10
Figure 2.3	The location of *Glyceraldehyde-3 Phosphate Dehydrogenase(GAPDH)* in chromosome 12	11
Figure 4.1	Total RNA extraction from NP69 and HK1 cell lines	20
Figure 4.2	Agarose gel electrophoresis of PCR amplification *RPL14* in NP69 cell line	22
Figure 4.3	Agarose gel electrophoresis of PCR amplification *GAPDH* in HK1 cell line	23
Figure 4.4	PCR Amplification of *RPL14* and *GAPDH* in NP69 and HK1	24
Figure 4.5	Result sequencing for *RPL14* using BLASTN	26
Figure 4.6	Result sequencing for *GAPDH* using BLASTN	27-28
Figure 5.1	The average of band intensity of PCR products in each gene in all cell line	31
Figure 9.1	The result of BLAST 2 sequences alignment for *RPL14*	40
Figure 9.2	The result of Primer-BLAST for *RPL14*	41
Figure 9.3	The result of Oligocalc that showed no secondary structure formation in forward primer for *RPL14*	41
Figure 9.4	The result of Oligocalc that showed no secondary structure formation in reverse primer for *RPL14*	42
Figure 9.5	The result of Oligo Analyzer that showed heterodimer analysis for *RPL14*	42

Figure 9.6	The result of Oligo Analyzer that showed hairpin analysis in forward primer for *RPL14*	42
Figure 9.7	The result of Oligo Analyzer that showed hairpin analysis in reverse primer for *RPL14*	43
Figure 9.8	The result of BLAST 2 sequences alignment for *GAPDH*	43
Figure 9.9	The result of Primer-BLAST for *GAPDH*	44
Figure 9.10	The result of OligoCalc that showed no secondary structure formation in forward primer for *GAPDH*	44
Figure 9.11	The result of OligoCalc that showed no secondary structure formation in reverse primer for *GAPDH*	45
Figure 9.12	The result of Oligo Analyzer that showed heterodimer analysis for *GAPDH*	45
Figure 9.13	The result of Oligo Analyzer that showed hairpin analysis in forward primer for *GAPDH*	45
Figure 9.14	The result of Oligo Analyzer that showed hairpin analysis in reverse primer for *GAPDH*	46
Figure 9.15	Verification of *RPL14* forward sequence using blast2q alignment	46
Figure 9.16	Verification of *RPL14* reverse sequence using blast2q alignment	47
Figure 9.17	Verification of *GAPDH* forward sequence using blast2q alignment	48
Figure 9.18	Verification of *GAPDH* reverse sequence using blast2q alignment	48

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is predominant cancer in Southern China and Southeast Asia which affect Cantonese inheritance. Previous research showed that ribosomal proteins (RPs) gene expressions in nasopharyngeal carcinoma (NPC) are differ from normal human nasopharyngeal epithelium cells with most RPs that are being down-regulated. Recent studies showed that disturbance of RPs might induce tumorigenesis. However, there is a lack of study done regarding *RPL14* specific investigation on NPC. Therefore, this study is proposed to extract Ribosomal Protein L14 gene (*RPL14*) to compare the differences on expression level in human nasopharyngeal cell lines (normal and carcinoma) by using a standard protocol, RNA was extracted by Trizol as starting material in this project and amplified using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with the aid of software Image Quant TL. This study revealed the PCR product with a length of 760 bp in *RPL14*. There is significant difference of *RPL14* expression levels in NPC.

Keywords : Nasopharyngeal carcinoma, Ribosomal protein gene, *RPL14*, RT-PCR and tumorigenesis.

ABSTRAK

Karsinoma nasofarinks (NPC) adalah kanser utama di China Selatan dan Asia Tenggara yang memberi kesan kepada masyarakat Kantonis. Kajian sebelum ini menunjukkan bahawa protein ribosom (RP) gen di dalam karsinoma nasofarinks (NPC) berbeza daripada sel-sel epitelium nasofarinks yang normal yang mana kebanyakan RP adalah di bawah proses regulasi. Kajian terbaharu menunjukkan bahawa gangguan RP mungkin mendorong kepada tumorigenesis. Walau bagaimanapun, kurangnya kajian yang dilakukan mengenai RPL14 terhadap siasatan tertentu pada NPC. Oleh itu, kajian ini mencadangkan untuk mengekstrak ribosom Protein L14 gen (RPL14) untuk membandingkan tahap perbezaan di dalam barisan sel nasofarinks manusia (normal dan karsinoma) dengan menggunakan protokol yang standard di mana RNA diekstrak dengan menggunakan Trizol sebagai bahan permulaan dalam projek ini dan diamplifikasi menggunakan Reverse Transcription Polymerase Chain Reaction (RT-PCR) dianalisis menggunakan perisian Image Quant TL. Kajian ini mendedahkan produk PCR dengan panjang 760 bp di dalam RPL14. Terdapat perubahan tahap ekspresi RPL14 di dalam NPC.

Kata kunci : Karsinoma nasofarinks, protein ribosom gen, RPL14, RT-PCR dan tumorigenesis.

1.0 Introduction

Gene expression is the most fundamental levels at which proteins or RNA products are expressed by performing its function through transcription and translation processes. Ribosome is one of the important organelles that functions in protein synthesis in all cells. It consists of two subunits that are ribosomal RNAs (rRNAs) and ribosomal proteins (RPs). RPs function as protein biosynthesis and many RPs also play roles as extraribosomal protein. Some believe that it involves in replication, transcription and repair, RNA splicing and modification, cell growth and proliferation, cellular transformation and regulation of apoptosis and development (Mao-De & Jing, 2007). Based on previous results, three ribosomal protein genes, *RPS26*, *RPS27*, and *RPL32* underexpressed were involved in nasopharynx cancer. Recent studies showed that disturbance of RPs might induce tumorigenesis.

Nasopharyngeal carcinoma (NPC) is a disease caused by malignant cell or tumour cell and is formed in nasopharynx tissue. Nasopharyngeal carcinoma (NPC) is predominant cancer in Southern China and Southeast Asia which affect Cantonese inheritance. In the previous study, NPC is a rare malignancy disease and misdiagnosed due to the nature of the disease itself (Cho, 2007). Therefore, the development of molecular biomarker is crucial for diagnosing in an early stage, monitor and give treatment to control the disease.

NPC is ranked as fourth cancer in Malaysia and most common cancer among men. Based on the data from National Cancer Registry of 2006, the age-standardised incidence rate (ASR) of the disease was 8.5 and 2.6 per 100,000 populations for males and females respectively. The disease was highlighted in Chinese population compared to other races. For Peninsular Malaysia, the ASR for NPC among Chinese males was 14 per 100,000 population and Chinese females was 3.8 per 100,000 population compared to 4 and 1.3 per 100,000 for Malay males

and females correspondingly. Krishna stated in 2004 that Indian males and females reported low incidence rate that was 1.0 and 0.2 per 100,000 populations respectively.

Two cell lines were used in this project to study the comparison of *RPL14* expression levels between normal and tumour cell from human nasopharyngeal epithelium. Cell lines were used instead of primary tumours because of their ready availability to detect differentially expressed protein, suitable condition and easy check for the reproducibility (Hay *et al.*, 1988).

RPL14 was reported to be increased in the presence of hepatocellular carcinoma (HCC). Alteration of *RPL14* as reported in squamous cell carcinoma. The expression of RPs in NPC is commonly seen as down-regulated although there are a few studies reporting up-regulated when expression of RP in NPC is overexpressed. In the previous study done by Huang *et al.*, 2006 and Shriver *et al.*, 1998 stated that the decreased expression of *RPL14* was observed in other carcinomas such as lung, oral and oesophagus. However, there is a lack of study done regarding *RPL14* specific investigation in nasopharyngeal carcinoma (NPC). The development of suitable marker is important and essential in NPC for early detection, genetically prone to certain disease in the early treatment (Cho, 2007). Thus, further studies on the analysis of *RPL14* expression level are necessary to compare the differences on expression level in human nasopharyngeal cell lines (normal and carcinoma).

The objectives of this study are:

1. To detect the expression of *RPL14* in normal human nasopharyngeal epithelial cell line, NP69 and tumour nasopharyngeal epithelial cell line, HK1.
2. To compare expression levels of *RPL14* gene between normal cell line and NPC cell line.

The study revealed the comparative expression levels of *RPL14* gene in normal nasopharyngeal epithelial cell line and nasopharyngeal carcinoma cell line with estimated of PCR product size of 760 bp.

2.0 Literature Review

2.1 Human Nasopharyngeal System

Nasopharyngeal system consists of three segments that are nasopharynx, oropharynx, and hypopharynx. Nasopharynx region is selected due present of nasopharyngeal carcinoma (NPC). The nasopharynx is a portion of pharynx which lies behind nasal fossae and extends inferiorly to the lower border of soft palate. It functions more on respiratory as collecting space where inspired air filters the impurities by lymphoid tissue (van Hassel & Gibb, 1991). Anita *et al.* stated that in 2006, nasopharynx is lined up with main pseudostratified columnar epithelium at birth and gradually transformed into stratified epithelium, non-keratinizing squamous epithelium except for transition zone.

2.2 Nasopharyngeal Carcinoma

2.2.1 Biology of NPC

Nasopharyngeal carcinoma (NPC) is a diverse type of head and neck cancer that refers to malignancy of nasopharynx tissue (Ma *et al.*, 2012). Besides, NPC is also an epithelium tumour which highest rate in Southern China and South East Asia specifically in Cantonese-Chinese population (Tao & Chan, 2007). NPC is endemic in Southern China due to genetic abnormalities and Epstein-Bair virus (EBV) infection is critical in pathogenesis of the disease (Chan *et al.*, 2002). In Malaysia, in the state of Sarawak, the native Bidayuh population was found to display highest age-standardized rates (ASR) of NPC occurrence in the world (Devi *et al.*, 2004). Figure 2.1 shows the location of the nasopharyngeal tumour.

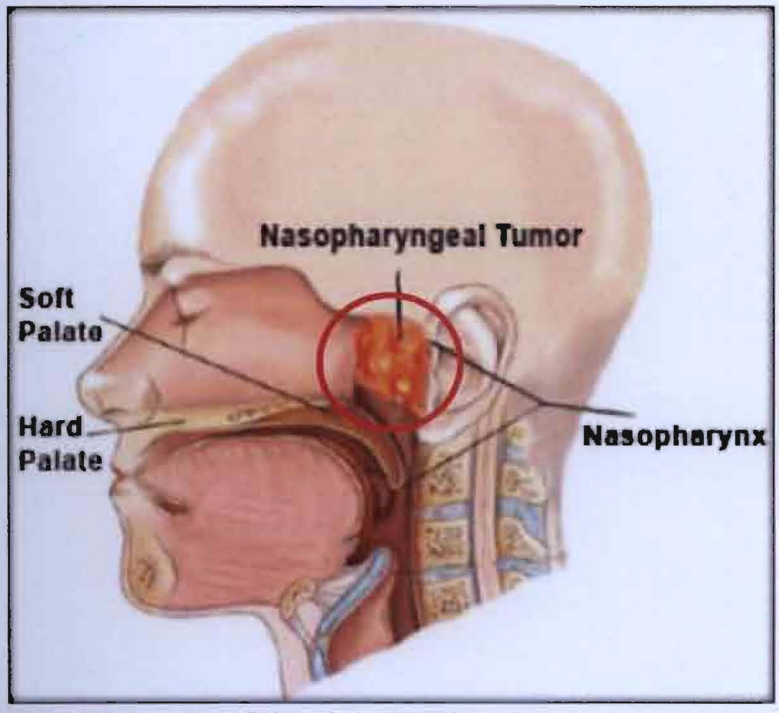

Figure 2.1. Location of Nasopharyngeal Tumour
(Ramsay Sime Darby Health Care, 2013)
Retrieved from: http://caringforcancer.areadigital.org/what-is-cancer/nasopharyngeal-cancer/

2.2.2 Histology of NPC

Nasopharyngeal Carcinoma is also known as an epithelial neoplasm. According to the World Health Organization (WHO), NPC is classified into three histopathological subtypes; Type I is the squamous cell carcinoma with distinct degrees of differentiation, Type II is non-keratinizing carcinoma where this tumour exhibit the maturation sequence characteristic of squamous cell carcinoma but no keratin formation and Type III is the undifferentiated carcinoma which made up of cells of various morphology (Adam *et al.*, 2014). Type III was frequently characterized as lymphoepithelioma because of the heavy infiltration of the primary tumour with lymphocytes WHO type II and III considered together as undifferentiated carcinoma of nasopharyngeal types (UCNT).

2.2.3 Etiology of NPC

80 % of all nasopharyngeal tumours from growth squamous cell arise in epithelial cell cancer at the walls of nasopharynx. The existence of NPC is multifactorial in origin and multigenic in mechanism (Sim *et al.*, 2008). Three major etiologic factors with causes NPC are genetic factor, an environmental carcinogen and Epstein–Barr Virus (EBV) infection. The genetic factor is one of the major etiological factors in NPC at which chromosome 3 is the most decisive genetic mutation where deletion on the short arm of chromosome 3 that is associated with the tumorigenesis of nasopharyngeal carcinoma (Chow *et al.*, 2004).

Secondly, environmental carcinogen have a large number of case-control studies in diverse population living in different parts of Asia (Southern China, Northern China and Thailand) and North America was confirmed that salt-cured fish containing large amount of nitrosamines carcinogenic substance that is taken from dietary food lead to NPC (Ning *et al.*, 1990). It is a belief that cooking these foodstuffs inhaled aerosolises carcinogenic nitrosamines.

Moreover, Epstein–Barr Virus (EBV) infection is proposed as one of the significant etiological factors in NPC (Sham *et al.*, 1997). The detection of EBV nuclear antigen and virus of DNA in NPC has discovered that EBV can infect epithelial cell and link to the transformation to cancer. About 90% of the adult population undifferentiated nasopharyngeal carcinomas (UNPC) have EBV positive by serology around the world. After primary infection at an early age, persistent EBV latent infection is found in some latent B cells but has not been detected in the nasopharyngeal epithelia of healthy individuals (Babcock *et al.*, 1998). EBV antibody titers higher are observed especially of immunoglobulin A (IgA) EBV-associated cancer in most NPC patients.

2.2.4 Human Nasopharyngeal Epithelium Cell Line (HK1 & NP69)

2.2.4.1 HK1

HK1 cell lines are well differentiated of squamous NPC cell lines that were established from a recurrent squamous carcinoma from a nasopharynx region of a Chinese male, patient which had radiation therapy for 17 1/2 years. It took over a year for long-term cell culture epithelioid cell line to be established after being passed for 72 times being cultured in RPMI-1640 medium supplemented with 15 % fetal calf serum to which penicillin and streptomycin were added by maintaining the cell lines. The cells have been shown by light and electron microscopies to prove it is the squamous epithelial type. The HK1 cell line was testing into the back of athymic nude BALB/c (nu/nu) mice and transplanted subcutaneously which makes tumours developed at the sites of inoculation, which on histological examination were shown to be well-differentiated squamous carcinomas, similar in morphology to the recurrent human tumour from which they were derived. Karyotypic analysis of cells from the cell line reveals that an aneuploid human type with a modal chromosome number of 74 with both numerical and structural aberrations (Huang *et al.*, 1980).

2.2.4.2 NP69

NP69 is an immortalized nasopharyngeal epithelial cell line established from primary non-malignant nasopharyngeal epithelial cells that had not been infected or modified by EBV. NP69 is also known as normal human nasopharyngeal epithelium. They are non-tumourigenic in nude mice and do not exhibit anchorage-independent growth in soft agar (Tsao *et al.*, 2002). This cell line can be a good model for investigating the biological properties of EBV genes including Latent Membrane Protein (LMP) 1. In their study, they demonstrated that LMP1 could induce many phenotypic changes in NP69 cells.

The gene expression profile of NP69 cells expressing LMP1 determined by using cDNA array analysis in the involvement of pathogenesis of NPC. It functions as a control for analysis of expression level of NPC cell line which is upregulated or downregulated. The NP69 cell line best cultured in culture medium RPMI1640 supplemented with 1 % dialyzed fetal bovine serum (Tsao *et al.*, 2002).

2.3 Ribosomal proteins and *RPL14*

2.3.1 Ribosomal protein (RP)

Ribosome is one of the major important cellular organelles for synthesizing a protein that consists of ribosomal RNAs (rRNAs) and ribosomal proteins (RPs) (Lai & Xu, 2007). Human ribosome consists of four rRNA species and 80 different RPs species. There are two subunits of ribosome that are large (L, 60S) and small (S, 40S) subunits. In addition, two ribosomal subunits combined with the accessory factor of protein that is protein associated with ribosome (PAR) to regulate its synthesis and recycling (Mao-De & Jing, 2007).

Cytogenetic map was successfully placed all 80 different RP genes on human genome in 2001. During evolution most of RPs conserved their amino acid sequence which are nearly identical among mammals will give their role in organism where most of the RPs are significantly conserved during evolution, their amino acid sequences are nearly identical among mammals, which suggests their important roles in organisms though they differ in their specific functions in the mature ribosome. Many ribosomal proteins (RPs) function in many roles that independent from protein biosynthesis that is called extraribosomal functions. Some researchers believed that most of the RPs improved their roles that were not necessity for the protein biosynthesis (Mao-De & Jing, 2007). RP genes give function in many roles that independent from protein biosynthesis that is called extraribosomal functions. Some researchers believed that

most of RPs improved their roles that were not necessity for the protein biosynthesis (Mao-De & Jing, 2007).

RP genes were always identified in screening the differentiated expressed genes of human diseases, especially in genetic diseases and cancers. The disturbances of their structures or expression levels were associated with various diseases. Ribosomal protein gene mutations or disturbance in their expression levels were identified in many inherited genetic diseases and cancers such as Diamond-Blackfan Anaemia Syndrome, Tuner Syndrome, Noonan Syndrome, Camurati-Engelmann Disease, Bardet-Biedl Syndrome (Yang & Liu, 2005).

2.3.2 Ribosomal Protein L14 (*RPL14*)

Ribosomal protein L14 is known as *RPL14* gene encodes a ribosomal protein, a component of 60S subunit. The human *RPL14* gene located at cytoplasm and map at chromosome 3p21 and it belongs to L14E family. It contains a basic region-leucine zipper (bZIP)-like domain (Shriver *et al.*, 1998). Transcript variant utilizing alternative polyA signals ad alternative 5'-terminal exons exist but all code the same protein [provided by (Refseq, 2008)]. Huang *et al.* in 2006 studied *RPL14* alter both genomic DNA and RNA level in oesophageal squamous carcinoma cell. This gene contains trinucleotide (GCT) repeat tract which means *RPL14* gene is highly polymorphic. Figure 2.2 show the location of *RPL14* in chromosome 3 in the red line.

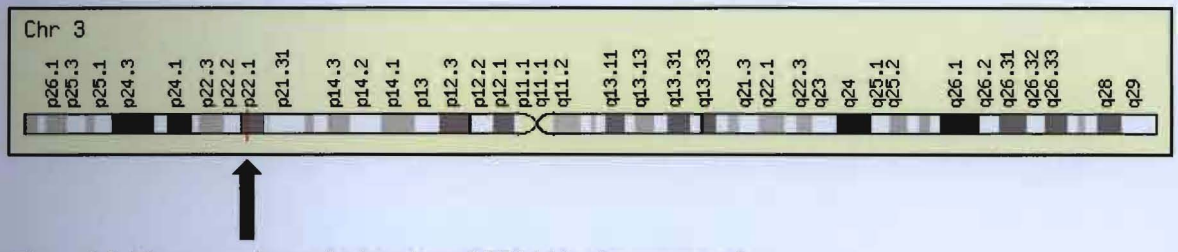

***Figure* 2.2**. The arrow shows the location of *RPL14* in chromosome 3.
Retrieved from http://www.genecards.org/cgi-bin/carddisp.pl?gene=RPL14

2.4 Housekeeping gene

2.4.1 *Glyceraldehyde-3 phosphate dehydrogenase (GAPDH)*

Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene is often used in many expression studies as a positive or loading control as it frequently stable and constitutively expressed at high levels in most tissues and cells (Nicholls *et al.*, 2012). It is also called as housekeeping gene. Previous studies showed that G*APDH* is highly expressed and stable in nasopharyngeal carcinoma and suitable for reference gene was used with similar expression level to normalize the target gene. It localized at chromosome 12 in human (Colell *et al.*, 2009) as shown in Figure 2.3.

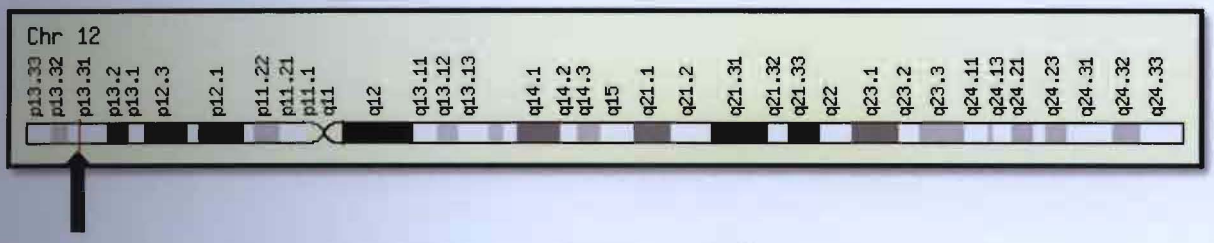

Figure 2.3. The arrow shows the location of *GAPDH* in chromosome 12.
Retrieved from: http://cdn.genecards.org/images/v4/genomic-location/GAPDH-gene.png

3.0 Materials and Methods

The list of materials and apparatus are shown in the table provided below.

Table 3.1. *Materials and reagent*

Materials and Reagents	Brand	Country of Manufacturer
Chloroform-Isoamyl Alcohol 24:1	Spectrum®	USA
DEPC-treated Water	Ambion®	Texas
dNTP Mix	Promega	USA
10 Kb GeneRuler™ DNA Ladder Mix	Fermentas	USA
Green Go Taq® Flexi Buffer	Promega	USA
Go Taq® DNA Polymerase	Promega	USA
6X Loading Dye	Fermentas	USA
Magnesium Chloride, $MgCl_2$	Promega	USA
Moloney Murine Leukaemia Virus Reverse Transcriptase	Promega	USA
Moloney Murine Leukaemia Virus Reverse Transcriptase Buffer	Promega	USA
Random Primer	Promega	USA
Rnase Rnasin Inhibitor	Promega	USA
TRIzol® Reagent	Invitrogen™	USA

Table 3.2. *Tools and apparatus*

Tools and Apparatus	Brand	Country of Manufacturer
ImageQuant™ TL Quant Software	GE Healthcare Life Science	USA
Polymerase Chain Reaction machine	SensoQuest GmbH	Germany
AlphaEaseFc software	Alpha Innotech Corporation	USA
Mikro 22R Centrifuge	Hettich Zentrifugen	Germany
Express Cool Turbo Freezer	LG	Korea
Microwave Oven	National	Japan
Polypropylene microcentrifuge tubes	Eppendorf®	UK
Power Pack™ Power Supply 3000	Major Science	USA
RNA Wood Hood	Köttermann	Germany
UV spectrophotometer	Ultrospec 1100 pro	UK
UV transilluminator	Wise UV. WUV-M10	UK
Weighing Machine	Setra	USA

3.1 Cell culture

Normal human nasopharyngeal epithelium cell line, NP69 and nasopharyngeal carcinoma cell line, HK1 was required for this purpose. Table 3.3 shows the sources of the cell line.

Table 3.3. Sources of cell lines

Designated name	Type of cell line	Sources
NP69	Immortalized human nasopharyngeal epithelium cell line (control)	Prof S.W. Tsao (Department of Anatomy, University of Hong Kong)
HK1	Nasopharyngeal carcinoma cell line	Faculty Medicine and Biological Health, Universiti Malaysia Sarawak

3.2 Total RNA Extraction

Total RNA extraction was extracted from the nasopharyngeal carcinoma (NPC) cell line, HK1 and normal human nasopharyngeal cell line, NP69 separately in a T25 flask by referring manufacturer protocol (Promega, USA). The cell media was discarded and the cell monolayer was rinsed with 990 µL of ice-cold PBS once. One millilitre of TRizol reagent (Invitrogen, USA) per T25 flask was added for homogenization state by passing three times through pipette to completely lyse the cell. The cell lysate (1 mL) were transferred into a sterile 1.5 mL centrifuge tube. The tube was vortexed thoroughly and incubated for 5 minutes at room temperature. 0.2 mL chloroform per 1 mL TRizol was added to separate the solution into aqueous phase, interphase and organic phase. The tube was vortexed for 15 seconds and incubated at room temperature for 2 minutes. Tube was centrifuged at 10,000 rpm for 15 minutes at 4 °C. Three distinct layers formed that are aqueous layer (RNA), interphase (DNA) and organic phase (protein). The aqueous layer which contains RNA was transferred into a new microcentrifuge tube and the remaining layers were discarded.

Next, 0.1 mL of isopropanol per 1 mL of TRizol was added that give function to form precipitates rapidly. The tube was incubated at room temperature for 10 minutes and centrifuged at 8,000 rpm for 10 minutes at 4 °C. Then, the supernatant was discarded. The pellet was washed once with 1 mL 75 % ethanol per 1 mL TRizol. The tube was vortexed and centrifuged at 5,500 rpm for 5 minutes at 4 °C. The supernatant was removed and the pellet was air-dried for 10 minutes and redissolved the pellet with 30 µL of nuclease-free water.

3.3 RNA Quantification and Quality Check

All required materials (P100 pipette and tips, autoclaved distilled water, waste beaker, Kimwipes, micro-cuvette for RNA, diluted samples, notebook and pen) were prepared beforehand. One microliter of sample with 49 µL of nuclease-free water (dilution factor 1:50) in a thin-walled PCR. UV-spectrophotometer (Ultrospec 1100 Pro, UK) switched on and waited for a few minutes for the equipment to start-up initialization. The necessary parameters were key in: wavelength= 260 nm, path length=10 mm, dilution factor= 50, units for concentration =µg/µL. Micro-cuvette was washed with distilled water twice and the waste was discarded into a waste beaker. The outer surface of micro-cuvette was wiped with Kimwipes tissue and blank sample was inserted into sample slot and was pressed for blank until the '0'reading shown. It is used to calibrate the instrument 100 % T (transmittance). The blank sample was removed and replaced with RNA sample and pressed the sample button. The reading value shown on the screen was recorded. The sample was discarded into a waste beaker and washed with distilled water.