Faculty of Resource Science and Technology

Gene Expression Analysis of Human Ribosomal Protein Genes, *RPeL13* and *RPeL14* in Nasopharyngeal Carcinoma (NPC) Cell Lines

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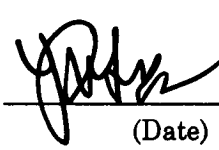
Student's signature  22/1/2018 Supervisor's signature: _____
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List of Abbreviations

AGE	**Agarose Gel Electrophoresis**
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
$MgCl_2$	Magnesium chloride
M-MLV	Moloney Murine Leukaemia Virus
NPC	Nasopharyngeal Carcinoma
RPeL13	Ribosomal Protein (eukaryotes) Large 13
RPeL14	Ribosomal Protein (eukaryotes) Large 14
RPs	Ribosomal Proteins
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
T_m	Melting Temperature
WHO	World Health Organisation
µg	Microgram
µl	Microliter
µM	Micromolar

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Gene Expression Analysis of Human Ribosomal Protein Genes, *RPeL13* and *RPeL14* in Nasopharyngeal Carcinoma (NPC) Cell Lines

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ABSTRACT

Human nasopharyngeal carcinoma (NPC) is a head and neck malignant tumor originating from the nasopharynx region. It is highly prevalent in Southern China and it is the fourth most common cancer in Malaysia. Recent studies have revealed that NPC are linked to ribosomal protein genes, particularly the large subunit ribosomal protein genes where it is found to have different expression patterns in NPC cell lines compared with normal human nasopharyngeal cell line. Hence, this research is conducted to study the gene expression of human ribosomal proteins genes, particularly *RPeL13* and *RPeL14* in normal human nasopharyngeal cell line, NP69 and nasopharyngeal carcinoma (NPC) cell lines, TW04, TW01, HK1, HONE1 and SUNE-1 by using two step Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). This study revealed that *RPeL14* transcript levels were detected in 2 out of 5 NPC cell lines, mainly TW01 and HONE-1 while no *RPeL13* transcript levels were identified in all six nasopharyngeal cell lines.

Key word: Ribosomal protein, nasopharyngeal carcinoma, RT-PCR, *RPeL13*, *RPeL14*

ABSTRAK

Karsinoma nasofarinks manusia (NPC) adalah tumor maglinan kepala dan leher yang berasal daripada kawasan nasofarinks. Penyakit kanser ini berleluasa di China Selatan dan merupakan kanser ke-empat yang paling kerap berlaku di Malaysia. Kajian kebelakangan ini telah menunjukkan bahawa NPC adalah berkait rapat dengan protein ribosom gen, terutamanya subunit besar protein ribosom gen yang didapati mempunyai corak gen ekspresi yang berlainan dalam garis sel NPC berbanding dengan garis sel normal epitelium nasofarinks. Jadi, kajian ini telah dijalankan untuk mengkaji ekspresi gen protein ribosom gen manusia, terutamanya RPeL13 dan RPeL14 dalam garis sel normal epitelium nasofarinks, NP 69 dan garis sel karsinoma nasofarinks TW01, TW04. HK1, HONE-1 dan SUNE-1 dengan menggunakan teknik dua langkah Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Dalam kajian ini, tahap transkrip RPeL14 dalam dua garis sel telah dikemukakan antara lima garis sel NPC terutamanya TW01 dan HONE-1. Akan tetapi, RPeL13 transkrip tidak dapat dikesan dalam enam garis sel nasofarinks.

Kata kunci: Protein ribosom, karsinoma nasofarinks, RT-PCR, RPeL13, RPeL14

1.0 INTRODUCTION

Gene expression is a fundamental cellular process that involves a complex staging interaction of organelles, factors and molecules to produce a functional product in the cell. It consists of two main stages: transcription and translation. The ribosome, which is a crucial translational machinery for protein biosynthesis in gene expression, is made up of ribosomal protein (RP) genes and rRNAs. Ribosomal protein genes had always been found to play a crucial role in ribosome assembly and protein synthesis. Until recently, studies showed that RPs carry out extra-ribosomal functions such as apoptosis, DNA repair and cell migration. Many studies frequently associated mutations of RPs with developmental disorders and human diseases such as Diamond-Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS) and the progression of tumorigenesis that leads to cancer development.

Interestingly, there is an increasing number of research showing that ribosomal proteins are linked to NPC. Nasopharyngeal carcinoma (NPC) is a subset of head and neck squamous cell cancers with unique endemic distribution and etiological factors (Mutirangura *et al.*, 1997). For many years, Epstein-Barr virus has been revealed to be an important factor for most nasopharyngeal carcinomas besides dietary and environmental reasons. Recent studies showed that there is strong evidence that genetic predisposition is involved in the genesis of NPC, among them are Human Leukocyte Antigen (HLA) gene, Glutathione S-transferase M1 (GSTM1), Polymeric immunoglobulin receptor (PIGR) and specific loci of chromosomal aberrations.

In ribosomal proteins, particularly from the large subunits in the ribosome have been shown to have different gene expression patterns in NPC cell lines, as compared to the normal nasopharyngeal cell line. For instance, Sim *et al.* (2016) found that

RPeL27, RPeL41, and RPeL43 genes are upregulated in NPC tumours. Another study by Hu (2010) has also examined an upregulation of *RPeL22* in her studies on NPC cell lines.

Despite numerous studies on NPC of the etiological causes of NPC, there has not been a significant evidence of these causes of the disease that correlates with NPC. One of the research problems of NPC is that 63.6% of the cases diagnosed are during its later stages (Azizah *et al.*, 2015). This is because the signs and symptoms are often mistaken to be common illness such as nasal congestion, headache and sore throat. Hence, the development of suitable marker is important and essential in NPC for early detection (Cho, 2007).

In this research, *RPeL13* and *RPeL14* genes was studied to identify their transcript levels of expression in NPC cell lines. Previous studies showed that these ribosomal protein genes are found to be linked with other types of cancers, yet it is one of the pioneering studies to be done in NPC. Hence, the objective of this research is to compare the gene expression patterns of *RPeL13* and *RPeL14* between normal nasopharyngeal cell line, NP69 and NPC cell lines of TW01, TW04, HK1, HONE1 and SUNE1.

2.0 Literature Review

2.1 Nasopharyngeal carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is one of the most common cancers originating in nasopharynx worldwide (Chen, 2015). It is a type of tumour arises from the surface epithelial lining situated at the upper part of the throat and behind the nostrils (Brennan, 2006), as shown in Figure 2.1.

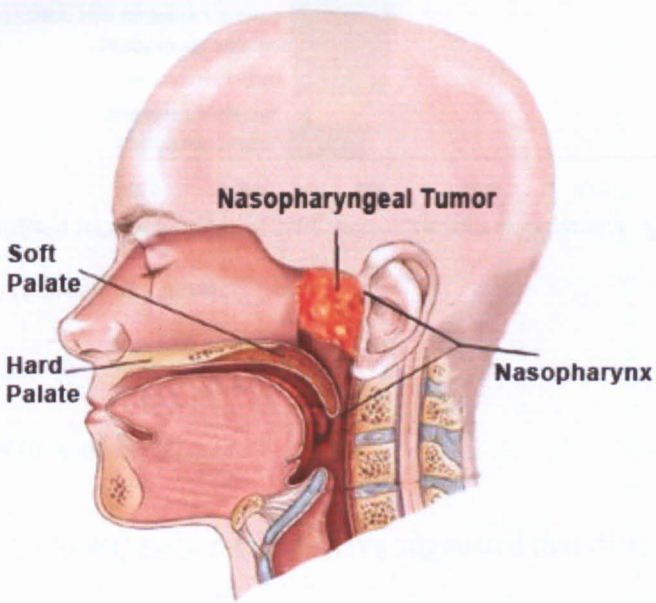

Figure 2.1: Origin of nasopharyngeal carcinoma (Yong, 2015).

NPC is a cancer that is particularly prevalent in South East Asia and Southern China (Yap, 2015). In general, men are more susceptible to NPC than women. Based on the National Cancer Registry Report 2007-2011, the lifetime risk for males is 1 in 143 whereas for females is 1 in 417 in Malaysia. Moreover, Tiong & Selva (2015) reported that in Peninsular Malaysia is found to be most susceptible to NPC while in East of Malaysia, it is the Bidayu ethnic group (69.9-70.8%). In fact, Devi & Pisani (2004) studies concluded that the Bidayu ethnic groups has one of the highest rates of NPC in worldwide as shown in Figure 2.2.

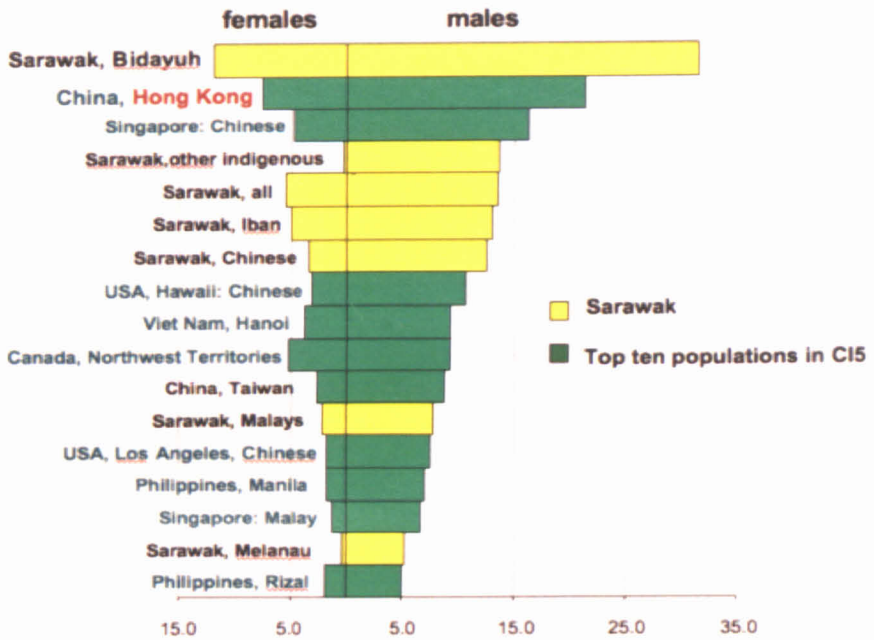

Figure 2.2: Age-standardised incidence rates of NPC across various populations in number of new cases per 100 000 population per year (Devi *et. al.*, 2004).

2.1.1 Etiological factors of NPC

Based on Gullo *et al.* (2008), earlier studies have suggested that dietary habits could be one of the major contributors to the oncogenic process. These include the ingestion of salted fish and preserved food that contain excessive amounts of carcinogenic compounds such as N-nitrosodimethyamine (NDMA), N-nitrospyrrolidene (NPYR) and N-nitrospiperidine (NPIP) (Gullo *et al.*, 2008). The exposure to smoke or chemical pollutants, including trace elements such as nickel have been reported to be associated with the development of NPC (Lu *et al.*, 2010). Numerous studies have also showed that NPC is closely related to Eptein-Barr Virus (EBV) infection. Young & Dawson (2014) explained that the EBV infected patients with NPC may appear to be a consequence of virus latency in the premalignant epithelial cells that have already undergone genetic changes. Thus, the etiology of NPC is highly associated with a complex interaction of genetic, environmental and dietary factors (Cho, 2007).

2.1.2 Histological classification

Since 1991, the World Health Organisation (WHO) had classified nasopharyngeal carcinoma into two subtypes: (Type 1) keratinised squamous cell carcinoma and (Type 2) non-keratinizing carcinoma. For Type 2, it is further subdivided into Type 2a, undifferentiated carcinoma and Type 2b, non-keratinizing undifferentiated carcinoma. However, studies showed that the distinction of NPC histological features can be unclear. In addition, the Chinese NPC classification has a more specific classification of NPC in comparison with the WHO classification. The consensus of NPC classification internationally still to be unified for better communication between researchers and medical officers (Wei *et al.*, 2011). Hence in 2005, the use of numerical designation of WHO types 1, 2 and 3 was eliminated.

The current WHO classification retains the terminology of the 1991 classification, with the addition of one category: basaloid squamous cell carcinoma (Barnes *et al.*, 2005). It is important to note that many studies published after that are still referring to the 1991 WHO NPC classification. According to Li & Zhong (2014), less than 0.2% of all NPCs are reported for basaloid squamous cell carcinoma in southern China. The NPC non-keratinizing carcinoma is the most common type, as stated by Brandon & Brenda (2013), in high-incidence areas they represent >95 % of all NPCs (compared with approximately 75 % of NPCs in low-incidence areas). It is also most common in endemic populations for Type 2, while the keratinizing and basaloid squamous cell carcinoma types tend to be more common in non-endemic populations (Bernard & Christopher, 2014). Nonkeratinizing NPCs also have a high association with positive EBV serologies, are more radiosensitive, and are more frequently associated with lymph node and distant metastasis (Brandon & Brenda, 2013).

2.2 Nasopharyngeal carcinoma (NPC) cell lines

To date, there have been more than 20 NPC cell lines that has been established since 1975 (Gullo, 2008). The NPC cell lines are usually characterized with their histological features and other properties such as EBV status, culturing method, patient's biodata etc. One of the first NPC cell line established was CNE-1, which is derived from a 58-year-old Chinese female with the histological type of well differentiated squamous cell carcinoma with a negative status of EBV (Zhang *et al.*, 1982). It is important to note that most of the NPC cell lines are EBV negative, some was initially positive but lo such as CNE-2 and SUNE-1. C666 is the only cell line that does not lose the EBV genome upon cell culture passaging. Moreover, one of the most recent NPC cell line established is NPC43 that harbours the EBV genome (Lin *et al.*, 2016). In this research, five NPC cell lines were being used namely TW 01, TW 04, HK 1, HONE 1 and SUNE 1.

2.2.1 TW 01

TW01, that was used to be named as NPC-TW039 is a NPC cell line derived from a 64-year-old Taiwanese male patient that had not been treated. The cell line established has a nature of moderately differentiated keratinizing squamous cell carcinoma. Karyotypic analysis showed the presence of multiple chromosomal abnormality. With the use of Giemsa banding technique, it is illustrated with a chromosomal number of 98 on average. According to Lin *et al.*, (1990), this cell line has the capability to produce a solid tum with 37 markers including translocation 21, deletion 7 and isochromosome 5, just to name a few (Lin *et al.*, 1990). This cell line has a capability of producing solid tumour mass when cells were transplanted to the nude mouse. However, TW01 does not exhibit oncogenic expression and has a negative EBV status.

2.2.1 TW 04

TW04 is a cell line derived from a 36-year-old Chinese male patient living in Taiwan. It has the histological feature of undifferentiated carcinoma and showed mild lymphocytic infiltration under microscopic examination. TW 04 had been passaged for more than 100 times and has a chromosomal number of 92 in average (Lin *et al.*, 1990). No EBV antigen was found in this cell line. Some of the special characteristics reported in this cell line is its overexpression of onco-myc and strong expression of vimentin (Lin *et al.*, 1993).

2.2.1 HK 1

HK 1 is established from a 59-year-old male Chinese patient with the histological feature of well differentiated squamous cell carcinoma. As mentioned by Huang and his team (1980). he was treated with radiation therapy in prior to the biopsy procedure and the tissue taken out was from a recurrent tumour. This cell line can be maintained in RPMI-1640 medium supplemented with 15 % fetal calf serum, to which 100 IU/ml penicillin and 100 & ml streptomycin PMI-1640. Based on the karyotype analysis, the cell line demonstrates an aneuploid with a modal chromosome number of 74 with both numerical and structural aberrations such as fragmentation, breakage, giant submetacentric chromosomes etc. HK1 cell line has a negative EBV status (Huang *et al.*, 1980).

2.2.1 HONE-1

HONE-1 is a NPC cell line derived from a 68-year-old Chinese male patient, showing histological features of poorly differentiated squamous cell carcinomas under microscopic examination. HONE-1 was initially detected as EBV positive but EBV-encoded nuclear antigen (EBNA) expression was loss after a few passages during the initial establishment of the cell line

(Glaser *et al.*, 1989). According to Strong and his team (2014) found that human papillomavirus 18 (HPV-18) was detected through RNA sequencing and real-time reverse transcription-PCR (RT-PCR) analysis in HONE-1. Further analysis of the chromosomal integration arrangement of HPV-18 in NPCs revealed patterns identical to those observed in HeLa cells. This finding showed that the early cultures of HONE-1 may be contamination by HeLa cells.

2.2.1 SUNE-1

SUNE 1 is derived from a female patient of poorly differentiated squamous cell carcinomas. Clones of SUNE-1 were subcultured, namely 5-8F and 6-10B. Both subclones have high and low tumorigenic and metastatic properties respectively.

2.3 Ribosomal protein genes

The human genome consists of 80 ribosomal protein genes, each with a single-copy gene (Rodnina, & Wintermeyer, 2011). These genes are active under every condition in every human cell type studied so far (Uechi *et al.*, 2001). They are crucial house-keeping genes that code for the architecture proteins in ribosomes. These RP genes are crucial house-keeping genes that code for the architecture proteins in ribosomes, the machinery responsible for protein synthesis (Rodnina, & Wintermeyer, 2011).

2.3.1 Ribosomal biogenesis

Ribosomal proteins are deemed not only as components of ribosomes, as since the last two decades, evidences showed that the also play fundamental roles in ribosome biogenesis (Xu *et al.*, 2016). Ribosomal biogenesis comprises the processing and folding of the pre-rRNA and its concomitant assembly with the ribosomal proteins (Kressler *et al.*, 2010). For instance, some

RPs function as RNA chaperones, stabilize rRNAs and promote their correct folding for the assembly of ribosomal subunits into a ribosome, whereas other RPs, such as *uS7*, *uS9*, *uS12*, *uS15*, *uL1*, and *uL18*, are responsible for direct contact with tRNAs (de las Heras-Rubio *et al.*, 2014; Wilson & Doudna, 2012). These studies showed that ribosomal proteins are crucial for cellular growth and maintenance.

2.3.2 Functions beyond the ribosome

Recent studies showed the roles of ribosomal proteins in the regulation of apoptosis, cell cycle arrest, cell proliferation, cell migration and invasion, and DNA damage repair (Xu *et al.*, 2016; Wang, 2015). One of the significant examples was the RP-MDM2-p53 pathway. Several RPs such as RPuL5 and RPeL15 were found to activate the p-53-dependent cell cycle arrest and apoptosis by directly interacting with MDM2 and inhibiting its E3 ubiquitin ligase activity towards p53 when in stress (Marval & Zhang, 2011; Wu *et al.*, 1993). Studies by Zhang & Lu (2009) further related human cancer with the mutations and inactivation of RPs in the p-53 pathway. One of the most recent studies showed that the deletion of ribosomal protein genes is a common vulnerability in human cancer especially involving with TP53 mutations. This situation may contribute to the risk of cancers (Ajore *et al.*, 2017).

2.3.3 Ribosomal proteins and cancer development

Ribosomal proteins such as *RPuL5* and *RPeL15* can activate pathways like the p53 system to induce apoptosis and cell arrest. However, studies had also shown that the perturbations of RPs were associated with the growth of tumours caused by nucleolar stress (Wang, 2015). Stress conditions in the nucleolus can be triggered by a variety of extracellular and intracellular insults that impair ribosomal biogenesis and function, such as chemicals, nutrient deprivation, DNA damaging agents, or genetic alterations (Zhang & Lu, 2009). It was conceivable that either

impairment or hyperactivation of the process was associated with deregulation of cell growth, which would eventually cause tumorigenesis, thus, leading to the development of cancers in tissues (Xu, 2016).

2.3.4 RP genes and NPC

Several studies had showed that mainly the large subunits of ribosomal protein genes were linked with NPC. One of the recent findings of NPC cell lines in relation with ribosomal proteins was done by Sim *et al.* (2016) showing the overexpression of *eL27*, *eL43* and *eL41* in NPC cell lines. Another study done by Yang and his team (2005) explained that *uL16* and *eL21* might have the same function in proliferation due to its upregulation in NPC cell line that may facilitate its survival during the metastasis of it to other targeted organs. *eL14* was also studied by Vasudevan (2015) in NPC cell line, HK1 and the results showed that there was no gene expression in *eL14*. By far, there have been no associated and limited studies showing the correlation of *eL13* and *eL14* with NPC respectively. The genetic basis that underlies the metastasis of nasopharyngeal carcinoma remains poorly understood. Hence, more research needs to be done for the characterization of the ribosomal protein gene expression patterns in NPC.

2.4 Ribosomal protein gene of interest

2.4.1 Ribosomal protein 13 (RPeL13) gene

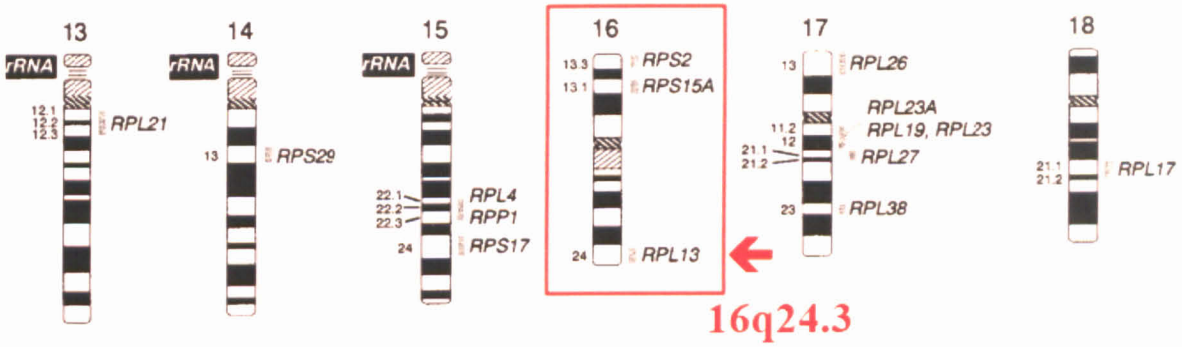

Figure 2.3: Gene loci of ribosomal protein gene, RPeL14 among other RPs (Uechi *et al.*, 2001).

The *RPeL13* is a gene located at 16q24.3 that encodes for 60S ribosomal protein *eL13* as shown in Figure 2.3. Several studies had been shown that there was a correlation between *eL13* gene expression with cancer. Kobayashi and his team (2006) observed that *eL13* was upregulated in gastrointestinal cancers. Two studies done by Kardos and his team (2013) & (2014) found out that *eL13* gene melanoma, cancers of the prostate, brain, lung, cervix, connective tissue, and bone. Although these studies suggested that e*L13* plays an essential role in the progression of some malignancies. Uterine cancers with high relative *RPeL13* tended to correlate with favorable survival, whereas prostate cancers with high *RPeL13* showed no differences in prognosis or clinical eatures (Dolezal *et al.*, 2017). While these studies showed the consistent upregulation trend of *RPeL13* in cancers, these evidences show that the role of *RPeL13* in different types of cancers are inconsistent in clinical correlations and needs further elucidation.

2.4.2 Ribosomal protein 14 (RPeL14) gene

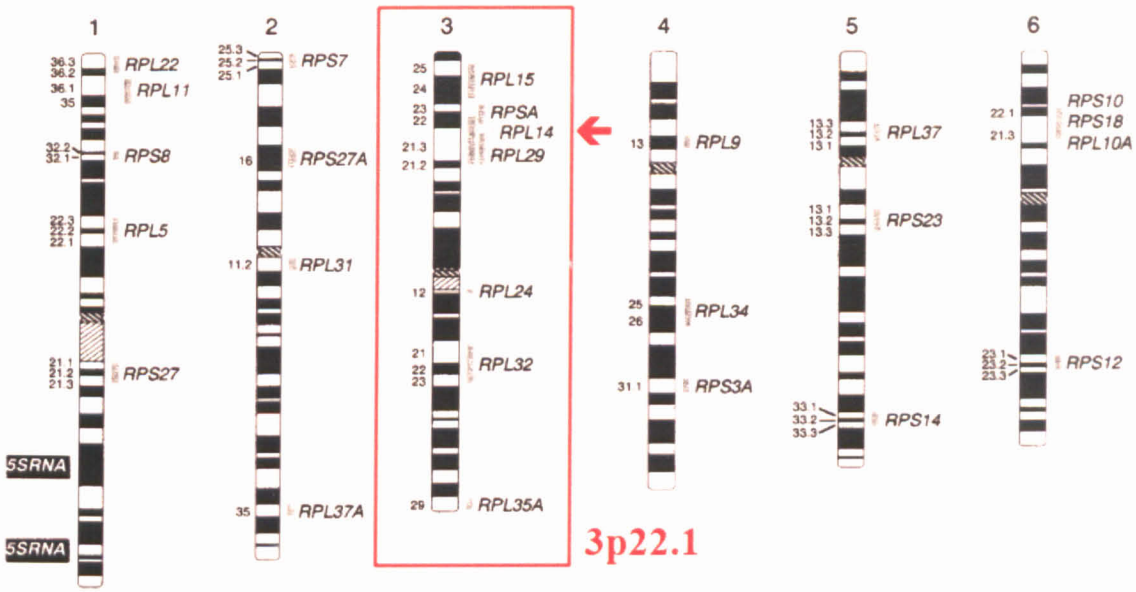

Figure 2.4: Gene loci of ribosomal protein gene, RPeL14 among other RPs (Uechi *et. al.*, 2001).

RPeL14 is a gene situated at chromosome 3, loci p21.3 that encodes for 60S ribosomal protein eL14 as shown in Figure 2.4. *RPeL14* contains a polymorphic CTG repeat encoding a polyalanine tract. According to Shriver *et. al.* (1998), polyalanine regions are found in a number of genes of developmental significance, consistent with the regulatory roles reported for ribosomal proteins. Shriver and his team (1998) found out that at the loci where the *eL14* gene is located at was consistently deleted in lung cancer, oral squamous cell carcinoma and renal cell carcinoma. Moreover, the loss of heterozygosity (LOH) regions of *eL14* was also frequently observed in primary breast tumours and were detected in breast cancer metastases (Aarøe *et al.*, 2010). Another study from Huang and her team (2006) showed that LOH of *eL14* was observed in 29 out of 68 (43%) tumours and decreased expression of the gene was detected in 31 out of 49 (63%) carcinomas. Hence, these results indicated that the deletion of *eL14* gene was correlated to a decrease of tumorigenesis. However, whether *eL14* gene suppresses cancers remains unclear and needs further elucidation.

2.5 Gene expression analysis – semi quantitative RT-PCR

The assessment of the gene expression profiles may explicate the molecular aspects of cancer progression. Thus, similarities and variations of gene expression profiles of cancer patients could be explored as potential biomarkers (Wei *et al.*, 2016). Quantitative measurement of specific gene expression is a critically important tool in understanding basic cellular mechanism and effects of various agents on cell function (Breljak *et al.*, 2005). RT-PCR is a modified version of PCR by using reverse transcriptase to convert RNA into cDNA and amplified in PCR. It is commonly used for its high sensitivity in quantification of low abundance nucleotide samples. RT-PCR allows researchers to quantify specific RNA transcripts and to detect variation in expression levels under different experimental conditions (Marone *et al.*, 2001).

2.4.2 Reference gene: GAPDH

A reference gene has an average expression that is independent of the biological process, treatment or disease that is studied (Vandesompele *et al.*, 2002). Vandesompele and his team (2002) stated that a housekeeping gene is assumed to be constant when experimental conditions affect tissue composition and gene expression levels simultaneously. And, Glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) gene is one of them. 12p13.31 10 exons gene is expressed constitutively and encodes for an enzyme that is involved in energy yielding step of glycolysis, DNA repair, membrane fusion and transport (Tristan *et al.*, 2011). Due to its nature, it is a housekeeping gene, has also been widely used as a housekeeping gene to normalize the expression of genes in studies of NPC and other types of cancers (Ma *et al.*, 2016). According to Tong and his team (2009), *GAPDH* is one of the most optimal reference genes for gene expression studies in nasopharyngeal carcinoma (NPC).