AN ASSOCIATIVE STUDY OF THE RIBOSOMAL PROTEIN GENES, RPS3 AND RPS12 WITH THE p53 TUMOUR SUPPRESSOR GENE

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An Associative Study of the Ribosomal Protein Genes, \( RPS3 \) and \( RPS12 \) with the \( p53 \) Tumour Suppressor Gene

First of all, I would like to extend my gratitude to my God who granted me all the grace and opportunity to complete this study. I also wish to express my utmost appreciation to my project supervisor, Associate Professor Dr. Edmund Sim Ui Hang for his unwavering guidance throughout the duration of my project. I hereby thank him for sharing his invaluable knowledge and giving me directions throughout the conduct of this final year project.

TING CHEE ENN

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Supervisor: Associate Professor Dr. Edmund Sim Ui Hang

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<tr>
<td>AGE</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>AMV</td>
<td>Avian myeloblastosis virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CIP1</td>
<td>COP1-interactive protein 1</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>GADD45</td>
<td>Growth arrest and DNA-damage-inducible 45</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double Minute 2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney murine leukaemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal cancer</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>RPL</td>
<td>Ribosomal protein large subunit</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RPS</td>
<td>Ribosomal protein small subunit</td>
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<tr>
<td>RPS12</td>
<td>Ribosomal protein small subunit 12</td>
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<tr>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td><strong>RT-PCR</strong></td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<tr>
<td><strong>SDS-PAGE</strong></td>
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<tr>
<td><strong>snoRNA</strong></td>
<td>Small nucleolar RNA</td>
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<tr>
<td><strong>Tm</strong></td>
<td>Melting temperature</td>
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<tr>
<td><strong>tRNA</strong></td>
<td>Transfer RNA</td>
</tr>
<tr>
<td><strong>μg</strong></td>
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<tr>
<td><strong>μl</strong></td>
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VII
An Associative Study of the Ribosomal Protein Genes, RPS3 and RPS12 with the p53 Tumour Suppressor Gene

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ABSTRACT

Ribosomal proteins (RP) are canonically associated with ribosome biogenesis. However, ribosomal protein small subunit 3 (RPS3) is also known for its DNA repair and apoptosis inducing ability whereas ribosomal protein small subunit 12 (RPS12) is recognized for its RNA splicing and modification functions. Furthermore, RPS3 was found to interact with a tumour suppressor gene, p53 through its KH domain. However, there has been no literature to date on the association between RPS12 and p53. In addition, RPS3 and RPS12 have been found to be associated to a number of cancer types although studies in nasopharyngeal carcinoma (NPC) have not been fully undertaken. In this study, correlative expression patterns of RPS3 and RPS12 to p53 was studied in nasopharyngeal cell lines. Correlation of RPS3 and RPS12 was also studied in normal and NPC cell lines. This was done using reverse transcriptase PCR (RT-PCR) for transcript analysis. This study revealed specific PCR products with estimated size of 172bp, 741bp and 1306bp for RPS12, RPS3 and p53 respectively. RPS3 and RPS12 were found to be significantly down-regulated (p < 0.05) in HK1 cell line suggesting association of these genes with well-differentiated nasopharyngeal carcinoma. On the other hand, no significant correlation (p > 0.05) was found between RPS3 and p53; RPS12 and p53 and also between RPS3 and RPS12 in all NPC and normal NP69 cell lines.

Keywords: Ribosomal proteins, RPS3, RPS12, p53, KH domain, RT-PCR

ABSTRAK

Protein ribosom (RP) telah dikaitkan dengan penghasilan ribosom. Walau bagaimanapun, protein ribosom subunit kecil 3 (RPS3) juga dikenali dengan keupayaan membaiki DNA dan mendorong apoptosis. Protein ribosom subunit kecil 12 (RPS12) dikenali dengan fungsi menyambat RNA dan mengubahsuaikan RNA. Tambahan pula, RPS3 didapati berinteraksi dengan gen penindas tumor p53 melalui domain KH. Walau bagaimanapun, tiada kajian dibuat antara RPS12 dan p53. Di samping itu, RPS3 dan RPS12 telah dikaitkan dalam beberapa jenis kanser walaupun kajian dalam karsinoma nasofarinks (NPC) tidak pernah dilaksanakan. Dalam kajian ini, corak ekspresi korelasi antara RPS3 dan RPS12 dengan p53 telah dikaji dalam sel nasofarinks. Korelasi RPS3 dan RPS12 juga dikaji. Analisis transkrip dilakukan dengan menggunakan "reverse transcriptase PCR" (RT-PCR). Kajian ini menghasilkan produk PCR dengan anggaran saiz 172bp, 741bp dan 1306bp untuk masing-masing RPS12, RPS3 dan p53. Dalam sel HK1, RPS3 dan RPS12 didapati diexpresi di bawah tahap biasa (p<0.05), mencadangkan hubungan gen-gen ini dengan karsinoma nasofarinks yang diberazakan. Sementara itu, tiada korelasi ketara (p>0.05) didapati antara RPS3 dan p53; RPS12 dan p53 dan juga antara RPS3 dan RPS12 dalam semua sel NPC dan sel biasa NP69.

Kata kunci: Protein ribosom, RPS3, RPS12, p53, domain KH, RT-PCR
1.0 INTRODUCTION

Nasopharyngeal cancer (NPC) is a rare type of cancer present in the head and neck region. The epidemiology of NPC is that it is rather unique to people in southern Asia (Chou et al., 2008). It is common among the Chinese population and also the Bidayuh population in Malaysia. Chou et al. (2008) stated that under the WHO classification system, there are 3 types of NPC. Type 1 NPC are those that are composed of keratinizing squamous cells; Type 2- non-keratinizing and Type 3- non keratinizing but less differentiated carcinoma. The disease is distinctively associated with Epstein-Barr virus (EBV) as most NPC tumors contain a high number of EBV (Chou et al., 2008). Additionally, exposure to nitrosamines in preserved foods is also listed as one of the risk factors of NPC (Chou et al., 2008).

Apart from the physical and environmental factors that are correlated to NPC, several genes are linked to NPC. Amongst these are the ribosomal protein (RP) genes. RPs were previously known to play a role in protein biogenesis, although increasing evidence are showing their involvement in extraribosomal functions such as RNA splicing and modification, DNA repair, regulation of cell growth and apoptosis, tumor suppressor gene and proto-oncogene regulation (Lai & Xu, 2007).

Around 80 or more RPs are found in human and they are divided into the RPL (Ribosomal Protein Large subunit) and the RPS (Ribosomal Protein Small subunit). Several RPS such as RPS3 and RPS12 are regarded as potential target for gene therapy. RPS3 for instance, have the DNA repair endonuclease function and also the apoptotic ability (Jang, Lee & Kim, 2004). On the other hand, RPS12 serve the function of RNA
splicing and modification. Moreover, RPS12 has also been correlated to colorectal, cervical (Zhang et al., 2011) and also gastric cancers (Zhang et al., 2012).

Interestingly, the tumour suppressor gene, p53 has been reported to correlate with RPs. The p53 tumor suppressor is negatively regulated by the MDM2 protein. RP has been shown to interact with MDM2, causing activation of the p53 and the increase of p53 level in the cells (Zhang & Lu, 2009). Specifically, RPS3 has been shown to interact with p53 and MDM2 protein (Yadavilli et al., 2009). RPS3 contains a specific KH domain that is known to co-activate p53 in response to DNA damage and able to bind DNA for the purpose of transcription (Yadavilli et al., 2009).

Despite of the interaction of RPS3 and p53, there are no proper studies yet on the interaction (if any) of RPS12 and p53. Moreover, the expression patterns of RPS3 and RPS12 has not been defined in nasopharyngeal cell lines. Our findings should provide fundamental insights into the molecular genetics of RPS3 and RPS12 with respect to NPC tumourigenesis.

Objectives of study are:

1. To investigate the expression pattern of RPS3 and RPS12 in nasopharyngeal cell lines.
2. To look into the correlation of RPS3 and RPS12 with the expression of p53 in NPC.
The hypothesis of this study was that there is a positive correlation between \textit{RPS3}, \textit{RPS12} and \textit{p53} such that the up-regulation of one gene will result in up-regulation of any of the three genes. Additionally, three genes are expected to be up-regulated in all three nasopharyngeal cell lines. The study aimed to investigate on the hypothesis by reverse-transcriptase PCR.
2.0 LITERATURE REVIEW

2.1 Ribosomal protein

Ribosomal protein (RP) genes are important as a prerequisite for ribosome production and also serve the purpose of protein synthesis (Ruggero & Pandolfi, 2003). Ribosomal proteins are also known for their extraribosomal functions such as cell growth or proliferation regulation, cell apoptosis regulation, tumour progression, RNA splicing and modification, DNA repair and many others (Lai & Jing, 2007). In short, extraribosomal functions of RPs may directly contribute to carcinogenesis (Lai & Xu, 2007).

In the past, RPs are thought to stabilize rRNA and to ensure the correct orientation of rRNA during ribosome biogenesis (Ruggero & Pandolfi, 2003). However, recent findings suggest new knowledge regarding specific domains of rRNA and new functions of RP. Ruggero and Pandolfi (2003) stated that RPs assist in the interaction between ribosome and mRNA and also interaction of initiation and elongation factor. In addition, many small nucleolar RNA (snoRNA) are found within the intronic region of RPs (Yoshihama et al., 2002). As snoRNA are a group of RNA that chemically modifies RNA such as tRNA and rRNA modification, this indicates the role of RP introns in protein synthesis. Furthermore, elements that control transcription are found in the RP gene intronic sequence. Therefore, new roles of intron in eukaryote gene expression are suggested to be correlated to RP genes (Yoshihama et al., 2002).
The roles of RPs in cancer are now more prominent. Many RPs that include both large (L) subunit and small (S) subunit are found to be differentially regulated in cancer cell lines (Sim et al., 2010) and also primary tumors (Ruggero & Pandolfi, 2003). Studies have also shown that mutation that occurred in RP genes lead to changes in cellular proliferation. Gene knockdown experiments on RPL16 gene have shown that the absence of RPL16 directs the total ceasing of ribosome production (Ruggero & Pandolfi, 2003). Moreover, mutation of RPL16 is also studied in model organisms such as Drosophila. The study observed smaller body size, recessive lethality and infertility in these flies (Ruggero & Pandolfi, 2003). All in all, it is shown that reduced RP will ultimately lead to down regulation of protein synthesis and reduced cell growth and proliferation.

2.1.1 Ribosomal Protein S3 (RPS3)

A functional study of RPS3 showed that RPS3 has distinct functions in DNA repairing and also apoptosis (Jang, Lee & Kim, 2004). Both functions are located within two separate functional domains which are the C-terminal DNA repair domain and the N-terminal apoptotic domain (Figure 2.1.1).

RPS3 is regarded as a determinant of cell fate (Jang, Lee & Kim, 2004). This is because the role of RPS3 in the activation of apoptosis is carried out through the activation of caspase-8 and caspase-3 (Jang, Lee & Kim, 2004). RPS3 also enhances the cellular sensitivity towards cytokine, which is required in the cytokine-induced apoptosis in nucleus.
Interestingly, RPS3 mutants which possessed N- or C-terminal deletions showed enhanced death-inducing ability (Jang, Lee & Kim, 2004). This further suggests that the apoptotic ability of RPS3 is activated by post-translational modification (Jang, Lee & Kim, 2004). Studies of RPS3 at the protein level will be pertinent in defining its function in tumourigenesis.
2.1.2 Ribosomal Protein S12 (RPS12)

RPS12 is a highly conserved protein across many species including bacteria, archaea and metazoans (Sharma et al., 2007). The protein has several highly conserved loops such as the PNSA and PGVR loops (Figure 2). RPS12 has been found to play a role in cellular growth progression via RNA splicing and modification function (Lai & Xu, 2007). RPS12 is also associated with the decoding of the ribosome (Sharma et al., 2007).

In addition, RPS12 has been shown to play a role in cancer. RPS12 has been regarded as cancer-related markers in colorectal tumor and also cervical squamous cell carcinoma due to its significant up-regulation in both types of cancer (Zhang et al., 2011). However, whether up-regulation of RPS12 is the causative factor of cancer or the consequence of cancer is yet to be uncovered.

Furthermore, the expression of RPS12 gene is increased in the precursor of adenocarcinomas – the adenomatous polyps, suggesting its role in cancer progression (Pogue-Geile et al., 1991). RPS12 gene is also significantly up-regulated in chronic atrophic gastritis (CAG), which is the precursor lesions of gastric cancer (Zhang et al., 2012). Moreover, RPS12 gene is also shown to be up-regulated in adjacent tissues of progressive cancers (Cheng et al., 2002). The up-regulation of the gene might act as a predisposition to promote cancer growth. More notably is that high expression of RPS12 gene is related to poor survival rate in gastric cancer patients, suggesting their role in regulating certain pathways or their direct relationship with tumor suppressor genes or oncogenes (Zhang et al., 2011).
2.2 p53- Tumor Suppressor Gene

Tumor suppressor p53 is a transcription factor which has the DNA binding function. The tumor suppressor ability is carried out by the consequent binding and activation of several other genes by p53. The p53 gene will bind and activate p21, WAF1, Cip1 and GADD45 in order to stop the cell cycle and repair the damaged DNA or correct the metabolic changes (Steele et al., 1998). Additionally, the apoptotic role of p53 is carried out by subsequent activation of Bax and IGF-BP3 which enhances apoptosis through blocking the activity of growth factors in order to halt mitotic events (Steele et al., 1998). The tumor suppressive activity of p53 is down-regulated by the mdm-2 pathway. MDM-2 serves to prevent the accumulation of p53 in the cells (Steele et al., 1998).
The cancer role of p53 is significant as more than 50 percent of the cancers have been correlated to mutations in p53 (Steele et al., 1998). As p53 also acts as a transcription factor, mutations occurring in the DNA binding domain lead to loss-of-function of the gene. Mutation of p53 thus allows damaged or mutated cells to survive and this further allows accumulation of mutation in tumor suppressor genes and proto-oncogenes leading to cancer progression (Steele et al., 1998). Interestingly, instead of the p53 mutation acting as a causative factor in cancer, there is suggestion that the accumulation of p53 may lead to mutation in the gene itself, which in turn eventually causes cancer (Steele et al., 1998).

2.3 Ribosomal Proteins and p53

Ribosomal proteins (RP) are correlated to p53 as they are linked to MDM2 binding. The activity of p53 is regulated by MDM2 proteins as they inactivate p53 (Zhang & Lu, 2007). RPs are regarded as a sensor that is responsible in sensing nucleolar stress and malfunction in ribosomes (Zhang & Lu, 2007). According to Zhang and Lu (2007), several RPs, including RPL5, RPL11 and RPL23 bind to MDM2, and thus indirectly activate the activity of p53.

Studies have also revealed an interaction between RPS3 and p53 in HEK293 cell line (Human embryonic kidney cell line). The KH domain in RPS3 is responsible for this interaction as it is needed for DNA damage response and transcription (Yadavilli et al., 2009). Knockdown of RPS3 in HEK293 cell line resulted in about 40% loss of p53 and also loss of 1 domain in MDM2 (Yadavilli et al., 2009). RPS3 also protects p53 by interrupting with MDM2's activity in the ubiquitination of p53 (Yadavilli et al., 2009).
2.4 Possible Correlation of RPS3, RPS12 and p53 in NPC

In colorectal cancer, RPS3 (Pogue-Geile et al., 1991) and RPS12 (Zhang et al., 2011) are found to be up-regulated. Furthermore, RPS3 has been reported to interact with p53 through its KH domain and a positive correlation of RPS3 and p53 have been observed in human embryonic kidney cell line (Yadavilli et al., 2009). In addition, p53 have been found to be highly expressed in NPC (Chow et al., 2008). Therefore, all evidences suggest that there is a possible correlation between RPS3, RPS12 and p53 in NPC.

2.5 Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase-PCR (RT-PCR) is a sensitive assay and can be used to compare the mRNA expression level between closely related genes, characterize mRNA expression pattern (Bustin, 2000) and thus provides a basis for functional studies of different genes. As gene expression studies are crucial to understanding of many human-related diseases, mRNA quantification that involves the transcript level is essential. For instance, expression studies of drug resistance marker (Ramachandran & Melnick, 1999), tumour stage assessment (Bustin & Dorudi, 1998) and expression studies of differentially regulated genes in NPC cell lines (Sim et al., 2010) all applied the RT-PCR assay to achieve their objectives.

The general procedure of RT-PCR involves the reverse transcription by the action of reverse transcriptase of viruses such as moloney murine leukaemia virus (M-MLV) and avian myeloblastosis virus (AMV). The reverse transcription will then create complementary DNA (cDNA) for the exponential amplification by DNA polymerases.
Elongation will then occur and new strand of DNA will be produced. In RT-PCR, choosing of primers is often correlated to the objective of amplification. According to Bustin (2000), using mRNA-specific primers can reduce background priming. On the other hand, by using oligo-dT primers, many mRNA molecules can be analysed when the amount of RNA is relatively small (Bustin, 2000).
3.0 MATERIALS AND METHODS

3.1 Cell Cultures

For this study, cell lines derived from normal and tumour human nasopharyngeal epithelium were used. The normal cell line NP69 (Tsao et al., 2002) is provided by Prof. Sai Wah, Tsao (The University of Hong Kong), two tumour cell lines (HONE1 and SUNE1) were from our collaborators in University Malaya, and tumour cell line HK1 was obtained from the Faculty of Medical and Health Sciences at Universiti Malaysia Sarawak respectively.

3.2 mRNA Expression Analysis

3.2.1 Total RNA extraction from cell lines

The extraction of total RNA from cell lines was done using Trizol reagent in combination with nuclease-free water, PBS, chloroform, Isopropyl alcohol and 70% ethanol. Firstly, the monolayer culture of the cells were rinsed with PBS and lysed by adding 1ml Trizol reagent to every 3.5cm diameter of flask. Sufficient Trizol reagent was ensured in order to prevent DNA contamination. The cells were scraped using cell scraper. Next, the cell suspension was transferred into a new microcentrifuge tube and incubated for 5 minutes at room temperature to dissociate nucleoprotein complexes in the sample.
Subsequently, 0.2ml of chloroform was added for every 1ml of Trizol reagent into the samples. The samples were vortexed vigorously and incubated for 5 minutes in room temperature. Then, at 4°C, the samples were centrifuged at 10000rpm for 10 minutes to separate the RNA from other components. After centrifugation, three distinct layers were observed and they were the lower phase containing phenol-chloroform, interphase, and the upper aqueous phase where the RNA was present. The RNA was extracted into a new centrifuge tube without touching the interphase and the volume of upper-aqueous phase was measured.

In order to precipitate the RNA, 1:2 ratio of isopropyl alcohol to Trizol reagent were used. The mixtures containing isopropyl alcohol and Trizol were mixed by inverting several times. The mixtures were incubated at 10°C for 10 minutes. Following this, centrifugations of the mixtures were conducted at 10000rpm at 4°C for 10 minutes. The supernatant was removed and 70% ethanol was used to wash the RNA pellets. The RNA samples were vortexed and centrifuged at 7500rpm at 4°C for 5 minutes. The RNA was pelleted and left to be air dried. Finally, the pellets were suspended in 30μl of nuclease-free water.
3.2.2 Agarose Gel Electrophoresis (AGE)

Following the RNA extraction, agarose gel electrophoresis (AGE) was conducted in order to check the quality of the RNA. 1% agarose were used. Before the AGE was conducted, RNA samples were mixed with 6X gel loading dyes by the ratio of 5:1. AGE was conducted at 90V for 25 minutes. After that, the gel was visualized using Alpha Digi Doc. (AlphaInnoTech, California) and gel photo was taken as a record.

3.2.3 Reverse Transcription (RT)

Reverse transcription (RT) was carried out by using M-MLV (Moloney Murine Leukimia Virus) Reverse Transcriptase (Promega, USA). A mixture of nuclease-free water (Promega, USA), 2μg/μl total RNA and random primers (Promega, USA) were heated to 70°C for 10 minutes and centrifuged. The mixture was then further mixed with 5X M-MLV Reaction buffer (Promega, USA), 10mM dNTP mix (Promega, USA) which also contains RNasin® Ribonuclease Inhibitor (Promega, USA). The mixture was gently mixed and kept at 42°C for 2 minutes. After that, M-MLV reverse transcriptase was added and incubated at 42°C for 50 minutes. RNase H (Promega, USA) was added following the heating of the mixture at 72°C for 15 minutes. Finally, the mixture was kept at 37°C for 10 minutes.