CHARACTERIZATION OF EXPRESSION PATTERNS OF RSPS, PIN AND CART GENES IN NORMAL AND TUMOUR HUMAN CELL LINES

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(30933)

Bachelor of Science with Honours (Resource Biotechnology) 2014
Characterization of Expression Patterns of *RSP3*, *PIN* and *CART* Genes in Normal and Tumour Human Cell Lines

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A Thesis Submitted In Partial Fulfilment of the Requirement of The Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Associate Professor Dr. Edmund Sim Ui Hang

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2014
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This declaration is made on the 24 day of June 2014.

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Date submitted Name of the student (Matric No.)

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<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>National Center for Biotechnology Information</td>
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<td>mRNA</td>
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<tr>
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<tr>
<td>M-MLV</td>
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<td>PIN</td>
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<tr>
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Characterization of Expression Patterns of RPS3, PIN and CART Genes in Normal and Tumour Human Cell Lines

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ABSTRACT

NPC is one of the common types of malignant disease in Southeast Asia, South China, North Africa and North America. RPS3 plays a role in the regulation of apoptosis and DNA repair. PIN is a critical catalyst that is associated in multiple oncogenic signalling pathways and CART is found to be an appetite regulator. These three genes have been reported to play a role in carcinogenesis. RPS3, PIN and CART were chosen as candidate genes due to previous in silico study predicting the association of PIN and CART genes with RPS3. However, the respective expression patterns of these genes in NPC cell lines have yet to be observed. The main objective of this study is to observe and to compare the expression patterns as well as to predict the co-relationship of these three genes in normal and NPC cell lines via RT-PCR. Relative quantification of RPS3 and PIN showed higher expression in SUNE1 and HK1 whereas lower expression in HONE1 as compared to that in NP69. For CART, undesired product (~100bp) was observed after PCR instead of the targeted gene of interest (~227bp), so subsequent gene analysis had to be suspended due to the problem stated. The results showed that up-regulation of RPS3 and PIN in SUNE1 and HK1 while down-regulation in HONE1 cell lines. However, the differential expression of RPS3 and PIN were insignificant (p > 0.05) in all NPC cell lines when compared to normal NP69 cell line.

Keywords: NPC, RPS3, PIN, CART, RT-PCR

ABSTRAK

NPC merupakan salah satu jenis penyakit malignant yang biasa dijumpai di Asia Tenggara, China Selatan, Afrika Utara dan Amerika Utara. RPS3 dikenali kerana berfungsi sebagai pengawal apoptosis dan membaiki DNA. PIN adalah pemangkin kritikal yang terlibat dalam pelbagai laluan isyarat onkogenik dan CART dikenalpasti sebagai pengatur selera makan. Ketiga-tiga gen ini dilaporkan berkaitan rapat dalam karsinogenesis. RPS3, PIN dan CART telah dipilih dalam kajian ini kerana kajian in silico yang terdahulu telah menunjukkan terdapat interaksi antara PIN dan CART dengan RPS3. Walau bagaimanapun, bukan eksperimental pada corak ekspresi gen yang tepat dalam sel NPC masih belum ditemui. Objektif utama dalam kajian ini adalah untuk memerhatikan dan membandingkan corak ekspresi gen serta mencari hubungan ketiga-tiga gen dalam sel normal dan NPC dengan menggunakan RT-PCR. Quantifikasi relatif untuk RPS3 dan PIN didapati telah diekspresikan lebih tinggi dalam sel SUNE1 dan HK1 manakala lebih rendah dalam sel HONE1 berbanding dalam sel NP69. Untuk CART, produk yang tidak dijangka (~100bp) telah diperhatikan selepas PCR manakala gen yang disasarkan (~227bp) tidak diperhatikan, analisis gen yang seluarnya telah diperhatikan. Hasil kajian ini menunjukkan bahwa RPS3 dan PIN diekspreskan dalam SUNE dan HK manakala pengurangan pengelompokan dalam HONE1. Walau bagaimanapun, ekspresi RPS3 dan PIN didapati mempunyai perbezaan yang tidak ketara (p > 0.05) dalam sel normal NP69 dan semua sel NPC.

Kata kunci: NPC, RPS3, PIN, CART, RT-PCR
1.0 INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous and squamous cell neoplasm and considered as one of the most common malignant disease in Southeast Asia, South China, North Africa and North America (Fang et al., 2012).

There are eighty types of ribosomal proteins (RPs) and four ribosomal RNA (rRNAs) species made up of human ribosomes (Lai & Xu, 2007). RP such as Ribosomal Protein small subunit 3 (RPS3) is linked to DNA repair and apoptosis, as well as up-regulation in colon adenocarcinomas and adenomatous polyps (Lee et al., 2010; Lim et al., 2002; Park et al., 2012). Peptidyl-Prolyl Cis/Trans Isomerases (PIN) acts as mitotic proteins regulators, involves in regulating mitotic progression, cell production, transformation, centrosome replication, overexpression in cancers and also reported to be associated with NPC risk (Lu et al., 2013; Shen et al., 1998; Suizu et al., 2006). On the other hand, Cocaine and Amphetamine Regulated Transcript (CART) serves the function in appetite regulator, cardiovascular function, bone modelling, mesocorticolimbic dopaminergic system as well as reported up-regulation in neuroendocrine tumors and small bowel carcinoids (Challis et al., 2000; Echwald et al., 1999; Landerholm et al., 2012; Miraglia del Giudice et al., 2006; Vicentic & Jone, 2007).

There is no proper studies and lack of experimental evidence on the gene expression levels of the RPS3, PIN and CART genes in nasopharyngeal and tumour cell lines. Thus, the reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out in order to carry out the gene transcript analysis. The findings should present the basic insight to the molecular genetics of the three genes in NPC tumourigenesis.
The objectives of this study are:

1. To observe the gene expression patterns of *RPS3*, *PIN* and *CART* in nasopharyngeal and NPC cell lines.
2. To make comparison to the level of the gene expression patterns between these three genes in both nasopharyngeal cell lines and NPC.
3. To predict the co-relationship of these three genes in normal and NPC cell lines.
2.0 LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is nasopharynx malignant tumour and occurs mostly in Southeast Asia, South China, North Africa and North America (Fang et al., 2012). Moreover, it has high capability to grow fast and invade the neighbour regions as well as metastasizes the regional lymph nodes and distant organs (Sun et al., 2012). Besides, it is affected by Epstein Barr Virus, environmental factors and inherited genes (Fang et al., 2012). Typically, NPC has three groups such that type I: typical keratinizing squamous cell carcinomas, type II: non-keratinizing squamous carcinomas and type III: undifferentiated carcinomas. According to the report, Southern China recorded there was about 2% type I patients, 3% type II patients and 95% type III patients (Fang et al., 2012; Sun et al., 2012). Figure below shows the anatomy nasopharynx and location of nasopharyngeal tumor.

Figure 1: Anatomy of nasopharynx (adapted from Ramsay Sime Darby Health Care, 2013.)
2.2 Ribosomal protein (RP)

There are eighty types of RPs and four ribosomal RNA (rRNAs) species that made up the human ribosome responsible for the protein synthesis. Besides, mutations or defects in the RP genes would implicate genetic disorders and affect gene expressions, so, the interruption in their structures or expression levels are correlated with multiple types of diseases. Besides for being important in protein productions, it enhances proper rRNAs folding during ribosomal gathering as well as aid in the relation between ribosome and mRNA. RPs are also crucial for extraribosomal functions for instance DNA repair, cell growth, apoptosis, cellular transformation and many others (Lai & Xu, 2007).

2.3 Ribosomal Protein S3 (RPS3)

*RPS3* (Gene ID: 6188) has four mRNA transcript variants: mRNA transcript variant 1 (NM_001005.4), mRNA transcript variant 2 (NM_001256802.1), mRNA transcript variant 3 (NM_001260506.1) and mRNA transcript variant 4 (NM_001260507.1) (Figure 2) (NCBI, 2014a). *RPS3* is located at the chromosome of 11q13.3–q13.5, and consists of 6115 base pairs which include 6 introns of 5330 base pairs and 7 exons of 785 base pairs (Lim et al., 2002; NCBI, 2014a). *RPS3* involves in 40S ribosomal subunit and has many functions that include translational processes, DNA repair and apoptosis (Yoon et al., 2011).

The *RPS3* also acts as a guide to apoptosis in DNA repair (due to the overproduction of *RPS3*) via AKT-mediated phosphorylation. It takes part in DNA repair activity in certain genome areas although it is hardly involved in large-scale of DNA repair. Mutations cause destruction of Akt-mediated, *RPS3* phosphorylation can eventually speed up apoptotic cell death. Changes in DNA repair system is due to the increment of the neuronal vulnerability (*RPS3* immunoreactivity was decreased) after several times of repeated restraint stress particularly in hippocampus (pyramidal cells) (Lee et al., 2010; Lim et al., 2002; Park et al., 2012).
There was up-regulation of $RPS3$ gene in colon adenocarcinomas and adenomatous polyps. (Lai & Xu, 2007) It has higher expression level in colorectal carcinoma as compared to normal colon tissues. The $RPS3$ mRNA is more in 7 out of 10 adenomatous polyps which are presumably in cancer progression (Pogue-Geile et al., 1991).

$RPS3$ also was reported to be correlated with $p53$ especially in human embryonic kidney cell line where high level of $p53$ was found may cause high risk of NPC development (Chou et al., 2008; Yadavilli et al., 2009). However, the up-regulation of $p53$ may bring the implications to $RPS3$ in NPC have yet to be linked with more experimental evidence.

![Figure 2: The four mRNA transcripts of RPS3 (adopted from NCBI, 2014a).](image)

2.4 Peptidyl-Prolyl Cis/Trans Isomerases ($PIN$)

Human $PIN$ (Gene ID: 5300) is located at C19p13, contains 4 exons and has a promoter region of about 1.5 kb and mRNA transcript variant 1 (NM_006221.3) (Figure 3) (Lu et al., 2013; NCBI, 2014b). $PIN$ encodes PPIs that catalyse the cis-trans peptide bonds N-terminal isomerisation to proline residues in polypeptide chains (Shaw, 2002). It acts as mitotic protein regulators that phosphorylates the Ser/Thr-Pro motifs and regulates mitotic progression. $PIN$ gene might also form complexes with other phosphoproteins and its concentration may change during the mitosis. In addition, phosphorylation on Ser/Thr-Pro motifs involves regulation of several important events which are cell production,
transformation and centrosome replication. Removal of some parts in PIN gene can significantly interrupt centrosome from duplicating without affecting DNA production (Shen, Stukenberg, Kirschner & Lu, 1998; Suizu, Ryo, Lim & Lu, 2006).

Studies showed PIN gene to be overexpressed in a variety of tumor tissues. It acts as a critical regulator that regulates numerous oncogenic and tumor suppressor proteins: cyclin D1, c-Jun, Bcl-2, b-catenin, p53 and so on (Lu et al., 2013). PIN was overexpressed in several types of cancer and there was an association between PIN polymorphism where two single nucleotide polymorphisms (SNPs) at positions -842G>C and -667T>C in the promoter region and the risk of NPC especially in Chinese populations. The -842CG genotype is linked to reduce the transcription of PIN and lower the risk of NPC (Lu et al., 2013). However, based on the evidence from meta-analysis, the results suggested that there are no significant associations between the PIN promoter (-667T>C) polymorphism and the risk of cancer where this polymorphism is not acts as a biomarker for cancer susceptibility (Tao et al, 2014). Further functional analysis should be carried out especially in nasopharyngeal cell to support the findings of PIN gene in NPC patients.

![Figure 3: PIN mRNA transcript variant 1 (adopted from NCBI, 2014b).](image-url)
2.5 Cocaine and Amphetamine Regulated Transcript (CART)

Human CART (Gene ID: 9607) span around 20kb, consists of 3 exons, 2 introns and mRNA prepropeptide (NM_004291.3) (Figure 4) (Dominguez, 2006; NCBI, 2014c). It is a vital appetite regulator (highly express in hypothalamus) and is involved in feeding, drug use, stress, cardiovascular function as well as bone modelling (Vicentic & Jone, 2007). Human CART protein is abundantly but distribute discretely in brain, pituitary and adrenal glands, pancreas as well as gut) takes part in appetite repressing activity which controlled by the CART gene. There were several pharmacological and behavioural evidences suggested that CART peptides modulate mesocorticolimbic dopaminergic system such as the movement, preservation and compulsion functions. In addition, CART system was speculated involves in other neurological disorders due to the dopaminergic transmission (Vicentic & Jone, 2007).

Previous research indicated that CART is expressed in multiple neuroendocrine tumours and small bowel carcinoids. In two different intestinal tumour cell lines, CART gene is found enhanced tumour cell growth capability regardless the patient age, disease stage, tumour grade or other presenting symptoms. The presence of CART peptide promotes the growth in small bowel carcinoid. Hence, the research indicated that CART gene expressed in small bowel carcinoid tumours is correlated with the worse survival (Landerholm et al., 2012). Further experimental evidence has yet to be linked with NPC scenario.

![Figure 4: CART prepropeptide mRNA (adopted from NCBI, 2014c).](image-url)
2.6 Reverse Transcriptase PCR (RT-PCR)

Polymerase Chain Reaction (PCR) was first described in 1985 and as one of the nucleic acid analyses. It amplifies specific DNA fragments which can result in millions of copies that can involve in multiple types of analyses. Semi-quantitative Reverse transcription (RT) reaction can be carried out before proceed to PCR. RT-PCR gives the opportunity to assess gene transcription in cells or tissues which the transcript level is vital for mRNA quantification. Besides, it analyses gene expression pattern in cells and tissues and compares the level of gene expression which gene expression studies are significant to study human gene-related diseases and tumour stage assessment. RT-PCR shows that total RNA extracted from cells or tissues is used as a substrate in RT reaction in order to produce cDNA that will be eventually as a template amplified by PCR (Santos, Sakai, Machado, Schippers & Greene, 2004).

The validity of the RT-PCR depends on the use of appropriate positive and negative controls. The positive control is used to give evidence that cDNA template is a good template for PCR amplification which the gene will be expressed in the cell line. On the other hand, the negative control is used to demonstrate the absence of template DNA in PCR amplification and the gene does not expresses in the cell line (Melo, Kent, Yan & Goldman, 1994). Hence, RT-PCR provides the experimental evidence for the gene expression pattern.

The general procedure of RT-PCR involves the RNA isolation from the cell or tissue followed by cDNA synthesis by RT. Then, PCR involves cDNA amplification by the process of denaturation, annealing and extension (Figure 5).
Figure 5. Schematic diagram of RT-PCR.
3.0 MATERIALS AND METHODS

3.1 Primer Design

The primers were designed by using the mRNA sequence of RPS3, PIN, CART and GAPDH retrieved from NCBI database. The accession numbers of the targeted genes were important to make sure the correct genes were taken and easy to be traced back. Then, the Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/; NCBI, 2009) was used to look for suggested primer pairs by pasting the accession number into the input field. Besides, the forward and reverse primers of the target genes were inserted into the input field of Primer BLAST to restrict and check for the other highly potential targeted genes or similar sequences. The forward and reverse primers were also checked by using OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html; Kibbe, 2007) for the potential hairpin formation, 3' complementarity and all potential self-annealing sites until the results showed all "none".

3.2 Cell Cultures

There were four cell lines: NP69, HONE1, SUNE1 and HK1 used in this project. HONE1 and SUNE1 are poorly differentiated NPC cell lines whereas HK1 is a well-differentiated NPC cell line (Fang et al., 2012; Sun et al., 2012). The table below shows the cell line name, type of cell line and their origin (Table 1).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of Cell Line</th>
<th>Obtained from</th>
</tr>
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<tbody>
<tr>
<td>NP69</td>
<td>Normal Human</td>
<td>Prof. Tsao Sai Wah from Department of Anatomy, University of Hong Kong</td>
</tr>
<tr>
<td></td>
<td>Nasopharyngeal Epithelial</td>
<td></td>
</tr>
<tr>
<td>SUNE1</td>
<td>Human Nasopharyngeal</td>
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<tr>
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<td>Human Nasopharyngeal</td>
<td>Faculty of Health and Medical Science, Universiti Malaysia</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>Sarawak</td>
</tr>
</tbody>
</table>

3.3 Total RNA Extraction from Cell Lines

First, the solution inside the cell flask was discarded and the cell monolayer was rinsed with 1ml of PBS solution (1 or 2 times). The solution was discarded after that. Then, 1ml of Trizol reagent per 3.5 cm diameter dish was added and the cell was scrapped by using cell scraper, followed by incubation for 5 minutes. Next, the cell lysate was passed for several times through a pipette and transferred to a new labeled microcentrifuge tube. After that, 0.2 ml of chloroform per 1ml Trizol reagent was added in microcentrifuge tube and the sample was vortexed vigorously for 15 seconds and incubated at room temperature for 5 minutes followed centrifugation for 1000rpm for 10 minutes at 4°C. Following that, the upper aqueous layer was transferred carefully without disturbing the interphase into a fresh microcentrifuge tube and 0.5 ml of isopropyl alcohol per 1 ml of Trizol reagent was added for
the initial homogenization. Then, it was centrifuged at 1000rpm for 5 minutes at 4°C and the
supernatant was removed completely followed by the addition of 1 ml of 75% ethanol per 1
ml of Trizol reagent. Next, RNA pellet was vacuum dried for 12 minutes in centrifugation
and air dried for 6 minutes. Lastly, the RNA was dissolved in 30µl of nuclease free water.

3.4 Agarose Gel Electrophoresis (AGE)

AGE was prepared in 1% as the target DNA has low molecular weight. First, 0.4 g of gel
powder was prepared in 40 ml of 1X TAE buffer (Amresco, USA) and stored in small
Erlenmeyer flask. The agarose gel then was heated for 1 minute and 10 seconds in microwave
oven (National, Japan). The gel was cooled and the comb was inserted. Following that, the
agarose gel was solidified by putting it inside the freezer (-20°C- 4°C; LG, Korea). The gel
was submerged in 1X TAE and mini-gels were used by loading the samples into the lanes
below the miniscus of the buffer solution. Next, the mixture was pipetted to the proper mini-
gel lanes, soon the power pack (Bio Rad, California) was turned on for 50 minutes, 100 V,
200A and started the gel electrophoresis. After that, gel was stained by EtBr solution inside
the plastic staining box and photographs was taken when using UV transilluminator (Vilber
Lourmat, France) in order to view EtBr-stained DNA.

3.5 Reverse Transcription

Firstly, Tube 1, 2 µg of RNA sample was added with 1µl of random primer and the mixture
was topped up to a volume to less than 15 µl in water. The mixture was incubated at 92 °C for
3 minutes in order to melt the secondary structure within the template and immediately
cooled on ice, then, the mixture was centrifuged briefly. Tube 2, 5 µl of M-MLV (Moloney