Expression Trend of Selected Ribosomal Protein Genes in Nasopharyngeal Carcinoma

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Abstract

Background: Ribosomal proteins are traditionally associated with protein biosynthesis until recent studies that implicated their extraribosomal functions in human diseases and cancers. Our previous studies using GeneFishing™ DEG method and microarray revealed underexpression of three ribosomal protein genes, *RPS26*, *RPS27*, and *RPL32* in cancer of the nasopharynx. Herein, we investigated the expression pattern and nucleotide sequence integrity of these genes in nasopharyngeal carcinoma to further delineate their involvement in tumourigenesis. The relationship of expression level with clinicopathologic factors was also statistically studied.

Methods: Quantitative Polymerase Chain Reaction was performed on nasopharyngeal carcinoma and their paired normal tissues. Expression and sequence of these three genes were analysed.

Results: All three ribosomal protein genes showed no significant difference in transcript expressions and no association could be established with clinicopathologic factors studied. No nucleotide aberrancy was detected in the coding regions of these genes.

Conclusion: There is no early evidence to substantiate possible involvement of *RPS26*, *RPS27*, and *RPL32* genes in NPC tumourigenesis.

Keywords: NPC, RP, RPS27, RPS26, RPL32, transcript expression

Introduction

Nasopharyngeal carcinoma (NPC) is a distinct type of head and neck cancer that refers to the malignancy of the nasopharynx tissue. NPC has its highest incidence in Southern China and South East Asia, and is more prevalent in the population of Cantonese-Chinese heritage (1). Interestingly, in the East Malaysian state of Sarawak, the native Bidayuh population was found to exhibit highest age-standardized rates of NPC occurrence in the world (2). Although many molecular studies have been carried out, NPC remains one of the most commonly misdiagnosed diseases due to the nature of the disease itself (3).

Thus, the development of a suitable biomarker is important and essential in the early diagnosis of the disease to better control the prognosis of the cancer.

Traditionally, ribosomal proteins (RP) are thought to play an important role mainly in catalysing protein translation. However, in 1996 extraribosomal functions of RPs was discovered (4). In a more recent review (5) a list of RPs associated with many extraribosomal functions that are independent of their own involvement in the protein biosynthesis was summarizes. Ribosomal proteins have been implicated in many human diseases and disorders. Gazda's group (6) reported association of *RPS19* with Diamond-

Blackfan Anemia, in which mutations of *RPS19* together with downregulation of other RP genes, alter transcription, translation, apoptosis and promote oncogenic pathways in the disease. In colorectal carcinoma, the differential expression of RP genes has been found (7,8). Studies by Amesterdam's group (9) using Zebrafish as model suggested RP genes to be candidate cancer causing genes. Developmental defects were also reported in RP knockdown Zebrafish (10). A recent study by MacInnes et al. (11) reported loss of *p53* synthesis in Zebrafish carrying heterozygous mutations for 17 different RP genes hence possibly predisposing Zebrafish to malignant peripheral nerve sheath tumours.

In our previous studies, *RPS26* and *RPS27* genes encoding proteins for small ribosomal subunit were identified to be downregulated in nasopharyngeal carcinoma (12). A subset of RP genes for the large ribosomal subunit was also found to be differentially expressed among cell lines derived from the human nasopharyngeal epithelium (13). Microarray screening on an NPC case also revealed differential expression of a few RPs that includes *RPL32* (unpublished data). This study was aimed at delineating possible involvement of *RPS26*, *RPS27*, and *RPL32* in NPC tumourigenesis, as well as determining the relationship of the expression levels of these RP genes with NPC associated clinicopathologic factors.

Materials and Methods

Tissue biopsies and total RNA extraction

Ethical approval for this study was provided by the Medical Research Ethics Committee (Ministry of Health Malaysia, Ref: (H) dlm. KKM/NIHSEC/08/0804/MRG-IMR). Biopsies of tumouric growths and their adjacent normal tissues were obtained via forceps-biopsy method from NPC suspects admitted to Sarawak General Hospital and Hospital Serian. These biopsy specimens were immediately kept in RNA later RNA stabilizing solution (Qiagen, USA) prior to total RNA extraction using Trizol method (Invitrogen, USA). Assessment of RNA quality and quantity was later performed via spectrophotometric analysis and gel electrophoresis. Only patients diagnosed as NPC cases were subjected to subsequent expression study. For each NPC subjects, the RNA extracted from the adjacent normal tissues would serve as controls. Both the NPC and normal tissues had been confirmed histopathologically by pathologists. The details of the NPC subjects in this study are as listed in Table 1.

Quantitative polymerase chain reaction (qPCR) analysis

Real-time quantitative PCR was carried out on eleven sample pairs. The extracted RNA was first DNase treated with RQ1 RNase-Free DNase

Table 1: Clinicopathologic details of NPC subjects in this study

Patient ID	Age	Gender	Ethnicity	TNM Staging	WHO Classification
GH20	36	M	Iban	II	III
GH22	44	M	Chinese	II	III
GH24	54	M	Iban	IV	III
GH27	58	M	Malay	IV	II
GH39	69	M	Malay	IV	III
GH41	68	M	Bidayuh	I	III
GH48	49	M	Malay	IV	II
GH54	61	M	Bidayuh	III	III
GH55	37	F	Lun Dayak	II	II
GH67	49	M	Bidayuh	III	III
HS96	56	M	Bidayuh	II	III

GH: Subjects admitted to Sarawak General Hospital, HS: Subjects admitted to Hospital Serian; M: Male, F: Female. Iban, Bidayuh and Lun Dayak are natives of Sarawak. TNM Staging: A cancer staging system that describes the extent of cancer in a patient's body based on size of tumour (T), whether regional lymph nodes are involved or not (N) and whether metastasis has occurred (M). Small, low-grade cancers with no metastasis and no spread to regional lymph nodes are classified as Stage I or II. High, large-grade cancers with spread to regional lymph nodes or organs are classified as Stage III, whereas Stage IV refers to cancers that have metastasized. WHO Classification: Classification by World Health Organization based on histopathological types. Type I - keratinizing carcinoma, Type II - non-keratinizing carcinoma, Type III - undifferentiated carcinoma.

(Promega, USA) and heat inactivated according to manufacturer's protocol. First strand cDNA synthesis was synthesized from 2 μg of total RNA using oligo-dT primers, catalysed by MML-V reverse transcriptase (Promega, USA) in a reaction volume of 25 μl. Then, 0.5 μl of the first strand cDNA was used as template for subsequent PCR amplification. A total of 4ng of cDNA was added to a final reaction volume of 25 μl containing 1X Rotor-Gene™ SYBR Green PCR Master Mix (Qiagen, USA) and 1 μM of each forward and reverse primer. All qPCR primers used in this study are listed in Table 2. The *β-actin* (14) and *RPS27* (15) qPCR primers are established primers. Singleplex amplification was then carried out in Rotor-Gene™ 6000 Rotary Analyzer (Qiagen, USA) with initial denaturation for 5 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 5 sec and annealing or extension at 60 °C for 10 sec. Changes in fluorescence of SYBR Green dye in every cycle were monitored with the aid of Rotor-Gene™ 6000 software version 1.7 (Qiagen, USA). The threshold cycle (CT) value for amplification of each gene was determined by auto threshold function of the software. Prior to the amplification, PCR efficiency and primers compatibility of gene of interest and reference gene were validated via standard curve method (16). Melting curve analysis with temperature rampling from 55 °C to 99 °C was also carried out in each run to confirm specificity of PCR amplification. *β-actin* which served as reference gene was used for normalization of the cDNA input. In each NPC cases studied, its respective normal controls served as calibrator. The ΔC_T value and relative

quantitative value $2^{-\Delta\Delta CT}$ were calculated (16) and statistical significance was later tested using $\Delta\Delta CT$ value (17). The experiment was carried out in duplicate.

Sequence analysis

RPS27 amplification was carried out in a mixture volume of 25 μl with final reaction concentrations of 1X GoTaq™ Reaction Buffer, 0.2 mM dNTPs, 3mM MgCl2, 1 μM of each *RPS27* specific primers (forward: 5'-ACGACCTACGCACACGAGA-3'; reverse: 5'-CACTCATCTTGACTCAGAGTGCT-3') and 1.25 U GoTaq® Flexi DNA Polymerase (Promega, USA). PCR amplifications were performed using PTC-2000 Peltier Thermal Cycler (MJ Research) converter. The mixture was first incubated at 96 °C for 1 min, followed by denaturation at 94 °C for 30 s and annealing at 64 °C for 30 s and then extension at 72 °C for 30 s. The complete amplification procedure was carried out for 30 cycles followed by further incubation at 72 °C for 5 min. On the other hand, amplifications of *RPS26* was carried out using 1 μM of each *RPS26* specific primers (18) at annealing temperature 54 °C. For the case of *RPL32* amplification, *RPL32* specific primers (forward: 5'-GTGGCAGCCATCTCCTTCT-3'; reverse: 5'-GAAAACGTGCACATGAGCTG-3') were used. Aliquots of PCR products were later size-fractionated by agarose gel electrophoresis. The amplicons ranged in size from 243-455 bp.

PCR products of the correct size were purified using Gel Extraction System extraction kit (Viogene, USA) according to manufacturer's

Table 2: qPCR primers used in real-time PCR expression study

Gene	Primer Sequence (5'-3')	PCR efficiency	r^2 of calibration curve
β-actin	F: GCCAACCGCGAGAAGATGA R: CATCACGATGCCAGTGGTA (14)	0.99	0.99944
RPS26	F: GCCGCAGCAGTCAGGGACAT R: GGCAGCACCCGCAGGTC AA	1.05	0.99265
RPS27	F: GTGAAATGCCCAGGATGCTATA R: TGTAGGCTGGCAGAGGACAG (15)	0.98	0.98496
RPL32	F: GAAGTTCCTGGTCCACAACG R: GCGATCTCGGCACAGTAAG	1.08	0.99521
p53	F: TCAACAAGATGTTTTGCCAACTG R: ATGTGCTGTGACTGCTTGTAGATG	1.03	0.99384
Paxillin	F: GAGGCTCGCGGCGGAAAAGT R: AGGGCGTCGAGGTCGTCCAT	0.96	0.98714

F: forward primer, R: reverse primer. Calibration curve: 5x dilution of cDNA input ranging from 40 ng to 0.064 ng except for *RPS27* which starts from 20 ng and ends at 0.032 ng.

instructions and then sent to a commercial sequencing service provider (1st Base Laboratory Sdn Bhd, Malaysia). All purified DNA samples were sequenced in both forward and reverse directions. All sequences obtained were verified by comparative analysis with the sequences in GenBank database (*RPS26* [GenBank: NM001029]; *RPS27* [GenBank: NM001030]; *RPL32* [GenBank: NM000994]). The sequence corresponding to the amplified gene or region of interest was searched using the Blastn program (http://www.ncbi.nlm.nih.gov) with the nucleotide sequence obtained as a query sequence. Verifications of forward and reverse sequences were performed using blast2q alignment tool available at the NCBI website.

Statistical analysis

Paired Student's t-test and Wilcoxon Signed Ranks test were used to test the significance of the difference in expression of gene of interest between the NPC cases and controls. Results were expressed as mean ± SD. Multiple Linear Regression (MLR) test was used to assess the association between demographic/clinicopathologic factors and expression of the RP gene(s). The correlation between *p53* or *Paxillin* expression and RP gene expression was calculated either with Pearson or Spearman correlation test. All statistical analyses were performed using SPSS® software version 17.0 (SPSS Inc., USA). Statistical significance was set as $P < 0.05$.

Results

Expressions of RPS26, RPS27 and RPL32 genes and their association with clinicopathologic factors

Real time qPCR analysis on 11 NPC cases namely GH20, GH22, GH24, GH27, GH39, GH41, GH48, GH54, GH55, GH67, and HS96 revealed that there was no significant difference in expressions of all 3 RP genes when comparing NPC cases to controls ($0.093 ≤ P ≤ 0.929$) (Table 3). Although statistically they are not significant, *RPS27* and *RPL32* did display a pattern of underexpression in 7 out of 11 cases (64%) and 8 out of 11 cases (73%), respectively. An association study using MLR analysis was performed to further investigate any possible hidden relationship between these RP genes and clinicopathogic factors. MLR analysis on NPC associated factors revealed no relationship between *RPS26*, *RPS27*, and *RPL32* expressions with age, ethnic group, TNM staging and WHO classification. There was also no linear relationship of each RP genes with each of the factors when we examined them at univariate level. The result was as shown in Table 4. The gender factor was not analysed because the ratio of cases was biased, M:F = 10:1.

Nucleotide surveillance of coding regions of RPS26, RPS27and RPL32

In order to detect presence of any genetic alterations that may be associated with NPC, full length cDNAs encompassing the entire coding regions of *RPS26*, *RPS27,* and *RPL32* were

Table 3: Expression of genes of interest in NPC cases and their paired normal controls ($n = 11$)

Gene	Expression Mean (SD)	*t* statistic (df)	*P* value
RPS26	N : -2.125 (3.7100) T : -1.250 (2.9800)	-0.089#	0.929
RPS27	N : -5.516 (6.5733) T : -4.079 (3.8269)	-0.606 (10)	0.558
RPL32	N : -6.012 (3.6969) T : -3.841 (1.5102)	-1.856 (10)	0.093
p53	N : 5.751 (3.0480) T : 4.846 (1.5555)	-1.129 (10)	0.285
PXN	N : 11.726 (2.9667) T : 10.470 (2.8300)	-1.036 (10)	0.324

SD = standard deviation, df = degree of freedom, N = normal controls, T = NPC cases, # Z statistic. Expression was calculated as $Δ C_T$ in which C_T of *β-actin* was subtracted from C_T of gene of interest. The C_T (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Thus, no unit is required. The smaller the value of $Δ C_T$ is, the higher the expression of a gene is. Statistical significance was determined using Paired Student's *t* test except for *RPS26*. In the case of *RPS26*, the distribution of data is skewed to the left. Thus, expression is reported as median and interquartile range (IQR) and analysed using Wilcoxon Signed Ranks Test.

amplified and sequenced. Not all samples used in the expression study were subjected to sequence analysis due to sample limitation. For *RPS26* and *RPL32*, sequence analysis was carried out on GH20, GH22, GH24, GH27, GH39, GH41, GH48, and GH55 whereas sequence analysis of *RPS27* was performed on all eleven samples. Off all the samples screened, no nucleotide aberrancy could be detected in the entire coding regions of the 3 RP genes studied.

Comparison of expression pattern: p53 and Paxillin Vs RP genes

The qPCR analysis for *p53* and *Paxillin* genes were carried out on sample GH20, GH22, GH24, GH27, GH39, GH41, GH48, GH54, GH55, GH67, and HS96. No significant difference was observed for both genes (Table 3). The relationship of these two genes with each RP genes was determined using Pearson's correlation test. For data spread that was not linear or that did not show bivariate normal distribution, Spearman's correlation test was used. For *p53*, correlations between *p53* and each RP genes were tested using Spearman's correlation test. No significant correlation was found ($0.298 \leq P \leq 0.979$). For *Paxillin*, Pearson correlation test was used except for the case of RS26. The analysis revealed that there was no significant correlation between *Paxillin* and each of *RPS27* and *RPL32* ($P = 0.977$ & 0.295, respectively). Spearman's correlation test on *RPS26* also showed that there was no correlation between the gene and *Paxillin* ($P = 0.689$).

Table 4: Simple linear regression analysis on NPC associated clinicopathologic variables with RP gene expression

Independent Variable	P value		
	RPS26	*RPS27*	*RPL32*
Age	0.919	0.893	0.735
Ethnic group	0.245	0.186	0.365
TNM staging	0.505	0.587	0.917
WHO classification	0.313	0.093	0.403

Expression was calculated as $\Delta \Delta C_T$ in which ΔC_T of normal controls was subtracted from ΔC_T of NPC cases. Ethnicity is categorised as (i) high risk group that includes Bidayuh (2) and Chinese (1) (ii) others that include Malay, Iban and Lun Dayak. TNM staging refers to cancer staging of the subjects eg. Stage I, II, III, and IV whereas WHO classification categorises the NPC tumours into Type I, II, and III based on the degree of differentiation.

Discussion

RPS26 had previously been implicated in type 1 diabetes (19) and Diamond-Blackfan Anemia (DBA) (19,20). Studies had shown that DBA is associated with predisposition to cancer in particularly acute myeloid leukemia and osteogenic sarcoma (21,22) and mutation of *RPS26* in DBA is common (19). In this study, however, we did not detect any nucleotide aberrancy in the entire coding region of *RPS26*. The mutation of *RPS26* therefore might not be a common event in some cancer types. Expression study and MLR analysis on qPCR data further confirmed that *RPS26* is unlikely to be associated with NPC tumourigenesis.

Overexpression of RPS27 or MPS-1 was reported previously in many different types of tumours and evidence had shown that it might involve in progression towards malignancy (23–28). Stack's group (29,30) using patients' serum protein in their studies, proposed that it could be a potential biomarker in the early detection and diagnosis of head and neck squamous cell carcinoma (HNSCC). In our study, we examined the expression of RPS27 at the transcript level using qPCR method and found that the expression is not significantly different in NPC cases relative to their paired normal controls. Since this current study incorporated more samples, our findings also invalidates our previous suspicion of the underexpression of RPS26 and RPS27 in NPC (12). Our findings also did not agree with findings by Stack's which reported RPS27 overexpression in HNSCC (26,30). In their study (30), they compared RPS27 level in the serum of 125 subjects and 89 controls. However, of the 125 subjects examined, only 4 were tumours of the nasopharynx and they were all from stage IV. Our study, which consists of eleven paired normal and NPC samples (that are difficult to come by), and incorporates tumours of all four stages, should therefore be more representative of the expression pattern of RPS27 in NPC progression. We could not establish any linear relationship between RPS27 and age and gender. Others had reported similar findings in breast and gastric cancers (24,27) although their data was based on protein level analysis and thus could not be compared directly with our findings from transcript level. Although studies in many cancers including HNSCC had shown that RPS27 had positive correlation with tumour grades and stages at the protein level (26–28), we did not observe such pattern in our study at

the transcript level. A recent study demonstrated that RPS27 was capable of reducing Paxillin mRNA and protein levels (31) in head and neck cancer cell line but the role of Paxillin remains unclear in HNSCC progression. Our expression analysis on Paxillin transcript did not reveal such trend in NPC cases studied. We are well aware that findings from protein level would have added meaningful insights to this study, but given the fact that the biopsy samples obtained were often less than 5mm3 in size, we did not have enough of the remains to perform any protein extraction or analysis after isolating the RNA. The absence of mutation in the coding region of the RPS27 gene further indicated that this RP gene might not be associated with tumourigenesis of NPC.

Differential expression of *RPL32* had been reported in prostate cancer cells and colorectal cancer (7,32). A recent study in *Schizosaccharomyces pombe* also suggested that *RPL32* might be a potential transcriptional regulator (33). In our study however, we could not find any evidence that support possible involvement of *RPL32* in NPC tumourigenesis. Expression and sequence analysis of the transcripts showed no significant differential expression and absence of nucleotide variation, respectively. No relationship could be established between clinicopathologic factors and *RPL32*.

Recently, some ribosomal proteins, such as *RPS3*, *RPS7*, *RPL11* and also our gene of interest *RPS27*, were reported to be able to regulate *p53* activity by binding to MDM2 (34–37). The *p53-MDM2* pathway is an important regulatory mechanism in the cells that could cause cell-cycle arrest or apoptosis. In order to investigate if there's any relation between the expressions of *RPS26*, *RPS27* and *RPL32* with *p53*, a correlation analysis was performed. No correlation was found between expressions of *RPS26* and *RPL32* with *p53*. We speculate that perhaps these 2 RP genes were not involved in the *MDM2-p53* pathway, or at least not in the way similar to *RPS3*, *RPS7,* or *RPL11*. As for *RPS27*, although Sun's group (37) reported transcriptional repression of *RPS27* by *p53* gene and that ectopic expression of *RPS27* protein could increase *p53* protein level, we could not detect any correlation between transcript expressions of these 2 genes in our study ($P = 0.977$, $n = 11$). However, it's worth mentioning that in an earlier, preliminary study by our group using reverse transcription PCR and with a larger sample size ($n = 19$), statistical analysis did show significant underexpression of this gene in NPC cases ($P = 0.034$, data not shown). Of these

19 samples, 9 were used in this current study: GH20, GH22, GH24, GH27, GH39, GH41, GH48, GH54, and GH55 but due to insufficient amount of RNA left, we could not perform qPCR analysis on all 19 NPC cases.

On overall, from our study, there is no evidence to show that *RPS26*, *RPS27,* and *RPL32* are involved in the tumourigenesis of NPC. It should be noted however that this study is based on a relatively small sample size ($n < 20$) and is carried out at the transcript level only. We focused on transcript expression because RP mRNA abundance is kept within a fairly narrow range in normal healthy cells and any variation in RP mRNA expression therefore may reflect possible involvements in extraribosomal functions (38). For the case of *RPS27*, although differential expression at protein level had been reported previously (26,30), we reported no differential expression of the transcripts. Such inconsistencies in findings arise perhaps as a result of the complexity of regulatory process of the ribosome biogenesis and also due to the limitation of our current knowledge on extraribosomal functions of RPs. It could also be for the reason that NPC is a distinct form of HNSCC cancer and thus the molecular pathogenesis pathway varies.

Conclusion

Expression study on *RPS26*, *RPS27,* and *RPL32* showed no differential expression in NPC cases. No relationship could be established between these RP genes with age, gender, ethnicity, cancer staging, and WHO classification. There was no correlation found between *p53* and all three RP genes studied. Absence of nucleotide aberrancy in the coding regions indicated that mutation of any of these RP genes might not be a common event in NPC tumourigenesis. Based on the sample size used, there is no empirical evidence that could suggest possible involvement of these three RP genes in the tumourigenesis of NPC.

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Competing interests

The author(s) declare that they have no competing interests.

Authors' Contribution

Conception and design, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, and collection and assembly of data: EUS, XMA, TYL, ASK
Conception and design, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, and statistical expertise: EUS, ASK, XMA, TYL, SKS, TST
Critical revision of the article for important intellectual content and statistical expertise: EUS, ASK
Provision of study materials or patients and administrative, technical, or logistic support: ASK, TST, SKS
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