The uS8, uS4, eS31, and uL14 Ribosomal Protein Genes Are Dysregulated in Nasopharyngeal Carcinoma Cell Lines

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The association of ribosomal proteins with carcinogenesis of nasopharyngeal carcinoma (NPC) has been established in a limited subset of ribosomal protein genes. To date, three ribosomal protein genes, eL27 (L27), eL41 (L41), and eL43 (L37a), have been found to be differentially expressed in cell lines derived from NPC tumors. This raises the possibility of more ribosomal protein genes that could be associated with NPC. In this study, we investigated the expression profiles of eight ribosomal protein genes, uS8 (S8), uS4 (S9), eS31 (S27a), eL6 (L6), eL18 (L18), uL14 (L23), eL24 (L24), and eL30 (L30), in six NPC-derived cell lines (HONE-1, SUNE1, HK1, TW01, TW04, and C666-1). Their expression levels were compared with that of a nonmalignant nasopharyngeal epithelial cell line (NP69) using quantitative real-time PCR (RT-qPCR) assay. Of the eight genes studied, the expressions of four ribosomal protein genes, uS8 (S8), uS4 (S9), eS31 (S27a), and uL14 (L23), were found to be significantly downregulated in NPC cell lines relative to NP69. Our findings provide novel empirical evidence of these four ribosomal protein genes as NPC-associated genetic factors and reinforce the relevance of ribosomal proteins in the carcinogenesis of nasopharyngeal cancer.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a distinct malignant form of head and neck cancer that originates from the lateral or posterosuperior mucosal epithelium of the pharynx. The World Health Organization (WHO) classifies NPC as three major histopathologic types: Type I, keratinizing squamous cell carcinoma; Type IIa, nonkeratinizing differentiated cell carcinoma; and Type IIb, nonkeratinizing undifferentiated carcinoma. Type IIb is the most common and contributes to 60% and 95% of NPC cases in North America and Southern China, respectively, and is associated with Epstein-Barr virus (EBV) infection [1]. Global incidence shows a pattern associated with geographical location, where the highest prevalence is among the Chinese population in Southeast Asia and Southern China [2]. Although the involvement of genetic factors in NPC carcinogenesis is widely known, the mechanisms of their influence and/or action in the disease are not fully understood. This could be largely due to the fact that the full range of NPC-associated genes is still unclear. Among some of these are the ribosomal protein (RP) genes.

The products of RP genes canonically function as major components of ribosomes during protein biosynthesis. However, in 1996, Wool [3] listed more than 30 potential extraribosomal functions of RPs that include apoptosis, DNA repair, RNA processing, and transcription regulation. Sequence mutation and differential expression of several ribosomal protein genes have been reported in many human congenital disorders and carcinomas. For instance, more than 200 distinct mutations in nine RP genes, namely, eS19 (S19), eS24 (S24), eS17 (S17), eS7 (S7), eS10 (S10), eS26 (S26), uL18 (L5), uL3 (L11), and eL33 (L35a), were identified in a majority of Diamond-Blackfan Anemia (DBA) cases [4–10]. Genetic lesions in eL22 (L22) that include heterozygous deletion and...
nucleotide mutation have been found in T-cell acute lymphoblastic leukemia and colorectal cancer, respectively [11, 12]. Besides structural aberrations, alteration in the expression pattern of RP genes has been found in many types of cancer. These include the deregulated expressions of \( uS3 \) (S3), \( eS19 \), and \( eS31 \) in colorectal cancer [13–15]; \( eL8 \) (L7a), \( eL19 \) (L19), \( eS27 \) (S27), and \( eL37 \) (L37) in prostate cancer [16–19]; \( eS27 \) in cells of breast tumor and gastric carcinoma; and \( eL5 \) and \( eL14 \) in ovarian cancer [20–22]. Since 2014, a new naming system for RP genes has been introduced to unambiguously identify each RP gene across broad taxonomic range [23]. We adhere to this new system in this paper but have also included the old names (in parentheses) at first mention of the genes.

Association of RP genes with NPC was initially reported for \( eS26 \) and \( eS27 \). These two RP genes were downregulated in tumors of NPC relative to normal controls [24]. Three other genes (\( eL27 \), \( eL41 \), and \( eL43 \)) were later proven to be NPC-associated RP factors in cell lines derived from NPC tissues [25, 26]. However, the hypothesis of \( eS26 \) and \( eS27 \) as NPC-associated RP genes was subsequently refuted [27]. Another RP gene, \( eL32 \) (L32), was also found to be not involved with NPC tumorigenesis [27]. This means that only a limited subset of RP genes are associated with NPC. The complete list of genes that belong to this subset is yet to be resolved. Here, we provide evidence of four more RP genes that are relevant to the context of NPC tumorigenesis. They are identified on the basis of significant differential expression pattern between NPC and nonmalignant nasopharyngeal epithelial cell lines.

2. Materials and Methods

2.1. Cell Lines and Cell Culture. Six NPC-derived cell lines (HONE-1 [28], SUNE-1 [29], HK1 [30], TW01 [31], TW04 [31], and C666-1 [32]) and an immortalized nonmalignant nasopharyngeal epithelial cell line (NP69 [33]) were used in this study. Many of these were originally sourced from the University of Hong Kong (laboratory of Professor George S. W. Tsao) with permission for use granted by the provider. The NPC cell lines were cultured in RPMI-1640 (Gibco, Life Technologies, USA) containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin (Gibco, USA). The EBV-positive cell line, C666-1, was cultured on fibronectin-coated (Sigma, USA) cell culture flask containing prewarmed (37 °C) RPMI-1640 medium. The NP69 cells were cultured in defined keratinocyte-serum-free medium (Invitrogen, USA) supplemented with 0.2 ng/mL growth factors, 5% heat-inactivated FBS, and 100 U/mL penicillin-streptomycin. All cells were maintained at 37 °C in a humidified environment containing 5% CO₂. Cells were harvested at a growth confluency of 70–80%.

2.2. Total RNA Extraction and Quantitative Reverse Transcription-PCR (qRT-PCR). Total cellular RNA was extracted from the cell cultures using TRIzol Reagent (Invitrogen, USA) according to the manufacturer’s protocol. The extracted RNAs were DNase-treated with RQI RNase-Free DNase (Promega, USA). First strand cDNA was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega, USA). Real-time PCR (or qPCR) was performed using Rotor-Gene™ SYBR Green PCR Kit (Qiagen, USA) and QuantiTect SYBR Green PCR Kit (Qiagen, USA) on a Rotor-Gene 6000 Rotary Analyzer (Qiagen, USA) and analyzed using Rotor-Gene 6000 software Version 2.3.3 (Qiagen, USA). For each assay, a total of 8 ng cDNA was added to a final reaction volume of 25 μL containing 1x Rotor-Gene SYBR Green PCR master mix or QuantiTect SYBR Green PCR master mix and 1μM of each forward and reverse primer. Table 1 lists the details of the PCR primers used for this study. Quantitative gene amplifications were performed using the following thermocycling conditions: initial denaturation for 5 minutes at 95 °C, 40 cycles of denaturation at 95 °C for 5 seconds, and annealing and extension at 60 °C for 20 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was used as endogenous control (or reference gene). Non-template reaction was used as negative control. Biological triplicate tests were done for all analysis.

The selection of the threshold intensity was set at a fixed intensity on the log-linear phase of the amplification curve for all the samples tested. Validation experiments, which included the generation of standard curves using a series of diluted cDNA samples, were carried out to ensure primer efficiency as well as target and reference gene amplification compatibility. Melt curve analysis and conventional agarose gel analysis were adopted alongside to verify the presence of a single amplicon. An interassay calibration scheme was adopted to minimize loading variation and to detect possible contamination with the inclusion of duplicate reactions and “no-template” control (NTC), respectively, in each qPCR assay. All samples were normalized to GAPDH as the endogenous control. Relative fold differences were calculated by the \( \Delta\Delta C_T \) method [34].

2.3. Validation of PCR Efficiency. Primer efficiency was validated by generating a duplicate fivefold serial dilution over five-log magnitude. A calibration curve for each gene was plotted with average quantification cycle (\( C_q \)) values against log input amount (4, 0.8, 0.16, 0.032, and 0.0064 ng/μL). PCR amplification efficiency was determined from the slope of the log-linear portion of the calibration curve as follows:

\[
\text{Amplification efficiency} = \left[ 10^{-\frac{1}{\text{slope}}} \right] - 1. \quad (1)
\]

Valid \( \Delta C_T \) calculation requires the PCR amplification efficiencies of the target and reference genes to be acceptably comparable. This was determined by evaluating the relative efficiencies of the target and reference amplification from the individually generated standard curves using the sample and log dilution. The \( C_{T \text{target}} - C_{T \text{reference}} \) values were plotted against log input amount of five-template DNA dilution (4, 0.8, 0.16, 0.032, and 0.0064 ng/μL). The slope of the resulting semi-log regression line (slope of \( C_q \) versus log of input amount) was used to determine the compatibility of the two PCR efficiencies, with the slope value (\( m \)) less than 0.1 taken as ideal. In this study, primer efficiency of the respective genes in an NPC cell line (HK1) was validated against the reference gene, GAPDH. Quality assessment tests indicated
that the efficiency curve for each of the primer sets relative to GAPDH was Correlation Coefficient ($R^2$) greater than or equal to 0.97 and PCR efficiency (or amplification compatibility) of at least 90% (within the ideal amplification range of 90–110%). Correspondingly, the validation plot of Δ$C_T$ versus log input amount of RNA shows $m$ values within the range of −3.6 and −3.1, thus ascertaining the validity of our qPCR experiments.

2.4. Statistical Analysis. A mean fold difference value of more than 1.0 was considered to be an overexpression while a fold difference value of less than 1.0 was taken as an underexpression. The difference of means (target genes expression expression pattern of each RP gene in NPC, the mean fold difference of each gene in the six NPC cell lines was averaged and compared to that of the normal (nonmalignant) nasopharyngeal epithelial cell line, NP69 (Table 3, Figure 1). Under-expression pattern is observed for all NPC cell lines except in HONE-1. Among the 8 RP genes tested, underexpression is statistically significant ($p < 0.05$) in all NPC cell lines relative to normal control for uS8, uS4, and uL14. In the case of eS31, significant downregulation is observed in all NPC cell lines except for HONE-1. Its upregulated pattern in HONE-1 is not statistically significant.

3. Results

3.1. Relative Expression Level of RP Genes in Each NPC Cell Line versus Normal Control. Fold difference of the 8 RP genes in each of the 6 NPC cell lines (HONE-1, SUNE-1, HK1, TW01, TW04, and C666-1) relative to the normal control (NP69) is shown in Table 2. When compared to that of the normal (nonmalignant) nasopharyngeal epithelial cell line, NP69 (Table 3, Figure 1). Under-expression pattern is observed for eS8, uS4, eS31, uL14, and uL24, with uS4 displaying the highest upregulation level (545.06-fold). However, only the downregulation pattern of uL24 is not statistically significant ($p = 0.054$). Overexpression trend is observed for eL6, eL18, and eL30. The expression of eL18 recorded the highest upregulation of 25.64-fold. Nonetheless, these overexpression patterns are not statistically significant. All in all, significant differential expression can only be concluded for eS8 ($p = 0.000166$), uS4 ($p = 0.023$), eS31 ($p = 0.00025$), and uL14 ($p = 2.47e^{-05}$). These genes show an underexpression pattern in NPC cell lines compared to normal cell line.
| Gene | Cell line | Fold difference ($2^{-\Delta\Delta Cq}$) | Std. deviation (SD) | $p$ value |
|------|-----------|----------------------------------------|--------------------|-----------|
| $eS8$ | NP69      | 1.012746                               | 0.011882           | 0.008019  |
|       | HONE-1    | 0.380311                               | 0.273122           | 2.34E-08  |
|       | SUNE-1    | 0.071494                               | 0.009687           | 0.000649  |
|       | HK1       | 0.251844                               | 0.163468           | 0.004816  |
|       | TWO1      | 0.402658                               | 0.444444           | 0.038129  |
|       | TWO4      | 0.253709                               | 0.282225           | 0.004816  |
|       | C666-1    | 0.196327                               | 0.085366           | 4.04E-05  |
| $uS4$ | NP69      | 1.21591                                | 0.314452           | 0.003026  |
|       | HONE-1    | 0.164457                               | 0.137157           | 0.009952  |
|       | SUNE-1    | 0.203615                               | 0.345601           | 0.004405  |
|       | HK1       | 0.149012                               | 0.225628           | 0.001452  |
|       | TWO1      | 0.032517                               | 0.028321           | 0.016465  |
|       | TWO4      | 0.416219                               | 0.29763            | 0.005476  |
|       | C666-1    | 0.216689                               | 0.22373            | 0.01062   |
| $cS31$ | NP69      | 1.013452                               | 0.010872           | 1.27319   |
|       | HONE-1    | 2.637661                               | 2.117301           | 0.158525  |
|       | SUNE-1    | 0.182123                               | 0.068557           | 0.005351  |
|       | HK1       | 0.560767                               | 0.239252           | 0.003531  |
|       | TWO1      | 0.415694                               | 0.235145           | 0.008531  |
|       | TWO4      | 0.246348                               | 0.160656           | 0.005588  |
|       | C666-1    | 0.322675                               | 0.329258           | 0.001523  |
| $eL6$ | NP69      | 1.080437                               | 0.043553           | 0.002428  |
|       | HONE-1    | 0.29751                                | 0.235938           | 0.010926  |
|       | SUNE-1    | 5.478057                               | 5.216626           | 0.073961  |
|       | HK1       | 10.04366                               | 8.672146           | 0.070656  |
|       | TWO1      | 2.92429                                | 1.74515            | 0.005374  |
|       | TWO4      | 6.398342                               | 4.51504            | 0.001523  |
|       | C666-1    | 2.365366                               | 0.344513           | 0.002133  |
| $eL18$ | NP69      | 1.02048                                | 0.005098           | 0.035128  |
|       | HONE-1    | 2.72834                                | 1.206147           | 0.015799  |
|       | SUNE-1    | 131.9168                               | 149.1636           | 0.10926   |
|       | HK1       | 3.345807                               | 2.137831           | 0.066337  |
|       | TWO1      | 1.467657                               | 0.354116           | 0.047003  |
|       | TWO4      | 1.900801                               | 0.691558           | 0.046081  |
|       | C666-1    | 12.5088                                | 6.650983           | 0.020133  |
| $uL14$ | NP69      | 1.123026                               | 0.152195           | 0.00872   |
|       | HONE-1    | 0.374748                               | 0.294766           | 0.002689  |
|       | SUNE-1    | 0.172974                               | 0.057157           | 0.004231  |
|       | HK1       | 0.305362                               | 0.199669           | 0.004133  |
|       | TWO1      | 0.189579                               | 0.093481           | 0.002221  |
|       | TWO4      | 0.308219                               | 0.190789           | 0.001187  |
|       | C666-1    | 0.171784                               | 0.184382           | 0.001372  |
| $eL24$ | NP69      | 1.058277                               | 0.07439            | 0.138828  |
|       | HONE-1    | 8.01882                                | 9.602711           | 0.02108   |
|       | SUNE-1    | 0.426845                               | 0.363781           | 0.262673  |
|       | HK1       | 1.807239                               | 1.8652             | 0.039945  |
|       | TWO1      | 0.504738                               | 0.403963           | 0.073365  |
|       | TWO4      | 0.573587                               | 0.461882           | 0.000299  |
Table 2: Continued.

| Gene | Cell line | Fold difference ($2^{-\Delta\Delta CT}$) | Std. deviation (SD) | p value |
|------|-----------|------------------------------------------|---------------------|---------|
| eL30 | NP69      | 1.072429                                 | 0.06969             | 0.097009|
|      | HONE-1    | 5.27733                                  | 4.672817            | 0.030566|
|      | SUNE-1    | 2.217279                                 | 3.50794             | 0.142807|
|      | HK1       | 21.05978                                 | 31.37735            | 0.165887|
|      | TWO1      | 8.75249                                  | 10.80404            | 0.177129|
|      | TWO4      | 5.414118                                 | 7.185418            | 0.177129|
|      | C666-1    | 27.56961                                 | 43.37632            | 0.174837|

Table 3: Relative expression of each RP gene in NPC cell lines compared to NP69. *Fold difference values < 1.0 were converted to linear-scale with the formula linear FD = (−1/FD).

| Gene | Cell line | Mean linear FD* | p value | Expression pattern |
|------|-----------|-----------------|---------|-------------------|
| eS8  | NP69      | 1.012746        | 0.000166| Significant underexpression |
|      | NPC       | −9.32115        |         |                    |
| uS4  | NP69      | 1.21591         | 0.023017| Significant underexpression |
|      | NPC       | −54.5064        |         |                    |
| eS31 | NP69      | 1.013452        | 0.00025 | Significant underexpression |
|      | NPC       | −3.34067        |         |                    |
| eL6  | NP69      | 1.080437        | 0.691146| Overexpression     |
|      | NPC       | 2.133808        |         |                    |
| eL18 | NP69      | 1.02048         | 0.07951 | Overexpression     |
|      | NPC       | 25.6447         |         |                    |
| uL14 | NP69      | 1.123026        | 2.47e−05| Significant underexpression |
|      | NPC       | −7.43259        |         |                    |
| eL24 | NP69      | 1.058277        | 0.053756| Underexpression    |
|      | NPC       | −3.74191        |         |                    |
| eL30 | NP69      | 1.072429        | 0.075329| Overexpression     |
|      | NPC       | 10.82959        |         |                    |

4. Discussion

In summary, our studies reveal the significant downregulation of three 40S RP genes (eS8, uS4, and eS31) and a 60S RP gene (uL14) in NPC cell lines. Our findings reinforce previous reports [25, 26] on the occurrence of dysregulated expression among a subset of ribosomal protein genes in NPC. The differential expression pattern of eS8, uS4, eS31, and uL14 is revealed for the first time in the NPC context and, hence, adds to the list of possible NPC-associated RP factors.

To date, differential expression of eS8 gene and its protein has been found in colorectal tumors/polyps [13] and colorectal carcinoma [35], respectively. However, its expression is constitutive and ubiquitous among normal and neoplastic thyroid tissues and cell lines [36–38]. This inconsistency of eS8’s expression characteristics across different types of cancer suggests its distinctive association with the type of malignant tissue/cell. More studies will be required to substantiate this. Molecularly, eS8 protein interacts with Cyclin Dependent Kinase 11B (CDK11B), a key mediator of Fas ligand-induced apoptosis [39]. It remains to be investigated whether downregulation of eS8 affects a disrupted eS8-CDK11B interaction and, thus, is associated with dysregulated cellular apoptotic pathway in NPC cells.

Differential expression of uS4 in colorectal cancer [40] is not consistently observed for human lung squamous cell, oral squamous cell, and liver cancer [41–43]. Similar to eS8, the association of uS4 with cancers is likely to be type-specific. Silencing of uS4 in glioma cells affects morphological differentiation without causing senescence [44]. In human embryonal carcinoma NTERA2 cells, uS4 is consistently underexpressed during retinoic acid induced neuronal differentiation [45]. It seems that there is relationship between the downregulation of uS4 and cellular differentiation. Our findings here corroborate with this notion. The level (number of fold differences) of uS4 underexpression in TWO4 and C666-1 cell lines is the lowest, followed by HONE-1 and SUNE-1, and the highest in HK1 and TWO1 (Table 2). TWO4 and C666-1 are undifferentiated cell lines, while HONE-1 and SUNE-1 are from poorly differentiated squamous cell carcinoma, and HK1 and TWO1 are from differentiated squamous carcinoma. The connection between uS4 expression and the degree of cellular differentiation warrants more research in order to be fully understood. At this stage, it implies that this RP can be a possible differentiation marker amenable for accurate histopathological analysis of NPC cells/tissues.

The eS31 is an ubiquitin C-terminal extension protein that is overexpressed in colorectal [15], breast [46], and renal [47]
The expression of eS8, uS4, eS31, and uL14 are significantly underexpressed in NPC cell lines relative to nonmalignant nasopharyngeal epithelial cells. These genes represent the latest addition to the limited list of RP genes that have association with cancer of the nasopharynx.

5. Conclusion

The ribosomal protein genes of eS8, uS4, eS31, and uL14 are significantly underexpressed in NPC cell lines relative to nonmalignant nasopharyngeal epithelial cells. These genes represent the latest addition to the limited list of RP genes that have association with cancer of the nasopharynx.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Edmund Ui-Hang Sim designed and supervised the study, interpreted the data, and wrote the manuscript. Kher-Lee Ng performed the experiments, analyzed the data, and wrote the manuscript. Choon-Weng Lee and Kumaran Narayanan aided in the interpretation of experimental data and the statistical analysis and cowrote the manuscript.

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