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Expression Analysis of Ribosomal Protein Genes, *eL13* and *eL14* in Nasopharyngeal Carcinoma Cell Lines

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ABSTRACT

Increasing evidence of the association between ribosomal protein (RP) genes with nasopharyngeal carcinoma (NPC) have been derived from findings of their differential expression patterns in NPC cell lines. Nevertheless, expression data from a comprehensive list of RP gene family members is still lacking. This paper reports the assessment of two RP genes, *eL13* and *eL14*, with regards to their expression patterns in several NPC cell lines (TW04, TW01, HK1, HONE1 and SUNE-1) relative to a non-malignant control (NP69). A conventional Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay was employed. Analysis of *eL13* has never been explored before this, whereas investigation of *eL14* represents an extended study. We found a general over-expression trend of *eL14* in 40% (2 of 5; TW01 and HONE-1) of the NPC cell lines studied, with higher upregulated level in only one (TW01) of them. However, this pattern of expression level is not statistically significant. Expression of *eL13* was not detected in any of the cell lines used. The inconsistency of these expression patterns demonstrates an elusive nature of RP activities in the malignancy of the nasopharynx.

Keywords: *eL13*, *eL14*, nasopharyngeal carcinoma, ribosomal protein, RT-PCR

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a subset of human head and neck squamous cell cancer with unique endemic distribution and etiological factors (Hildesheim & Levine, 1993), and represents a malignancy that arises from the surface epithelial lining at the upper part of the throat and behind the nostrils – the nasopharynx (Brennan, 2006). NPC is prevalent in Southern China and South East Asia with a higher incidence among the Chinese compared to other ethnicities, notably with the Cantonese dialect group (Armstrong, Kutty, Dharmalingam & Ponnudurai 1979; Li, Yu & Henderson, 1985). In the East Malaysian state of Sarawak, the Bidayuh community is the highest risk group (Devi, Pisani, Tang & Parkin, 2004; Sim, Tiong, Selva, Ong & Teh, 2010a; Tiong & Selva, 2005), with family history as a strong predictor (Yeo, Beh, Tan & Pei Jye, 2018). Etiological factors of NPC can include Epstein-Barr Virus (EBV) infection, genetic predisposition, dietary and other environmental influences (Chang & Adami, 2006). WHO Classification of NPC divides it into 2 major types: Type 1, the keratinizing squamous cell carcinoma; and Type 2, non-keratinizing carcinoma. Type 2 is further divided into Type 2a, undifferentiated carcinoma; and Type 2b, differentiated carcinoma. Type 2 is the most common and constitutes 95% of NPC cases in high-incidence areas (Peterson & Nelson, 2013). Type 1 is rare and more common in non-endemic populations (Li & Zong, 2014). Type 2 is associated with positive EBV serology, more radiosensitive, and frequently associated with lymph node and distant metastasis (Peterson & Nelson, 2013). A major concern of NPC is that 63.6% of diagnosed cases are discovered at later stages (Azizah, Saleha, Hashimah, Asmah & Mastulu, 2015). It remains one of the most misdiagnosed cancers because early signs and symptoms were often mistaken as common illnesses such as nasal congestion, headache and sore throat, hence the urgency for accurate early detection biomarkers (Cho, 2007). To facilitate closer biomolecular studies, NPC cell line models have been established since 1975 (Gullo, Low & Teoh, 2008).

For over two decades, the functions of ribosomal protein (RP) genes have been linked to activities beyond ribosome-mediated protein synthesis (Wool, 1996). These extra-ribosomal roles include apoptosis, cell cycle arrest, cell proliferation, cell migration and invasion, and DNA damage repair (Wang *et al*., 2015; Xu, Xiong & Sun, 2016). Their association and involvement with various cancers have been extensively discussed (de las Heras-Rubio, Perucho, Paciucci, Vilardell & LLeonart, 2013; Goudarzi & Lindström, 2016; Xu et al., 2016). In NPC, association of malignancy with specific RP genes is increasingly evident (Dolezal, Dash & Prochownik, 2018; Fang *et al*., 2008; Sim, Toh & Tiong, 2008; Sim, Ang, Ng, Lee & Narayanan, 2010b; Sim, Chan, Ng, Lee & Narayanan, 2016; Sim, Ng, Lee & Narayanan, 2017; Sim, Chee, Vasudevan, Ng & Chan, 2018; Yang *et al*.,

2005). Despite this, the functional significance of putative NPC-associated RP genes remains elusive due to inconsistency in their expression patterns among studies (Ma *et al*., 2012). This highlighted the complexity of their mechanism in tumor development and malignant transformation of NPC cells. The two RP genes studied herein are *eL13* and *eL14*, both encoding components for the 60S (large) subunit of the ribosome complex. *eL13* and *eL14* are found in chromosomal positions 16q24.3 and 3p21.3, respectively. Correlation between upregulation of *eL13* and cancers was demonstrated in gastrointestinal cancer (Kobayashi *et al*., 2006), melanoma (Kardos, Dai & Robertson, 2014), colorectal cancer (Takemasa *et al*., 2012; Xu *et al*., 2017). However, it remains relatively ubiquitous in clear-cell renal carcinoma irrespective of clinico-pathological characteristics (Wierzbicki *et al*., 2014). Expression behavior of *eL13* in NPC has not been reported in literature. Early evidence of *eL14*'s involvement in cancers was from observation of loss of heterozygosity (LOH) in the loci of *eL14* in the lung, oral squamous cell, and renal cell carcinomas (Shriver *et al*., 1998). This was also found in esophageal squamous cell carcinoma (He *et al*., 2007), and breast cancer (Aarøe *et al*., 2010; Huang *et al*., 2006). Interestingly, decreased expression of the gene was also observed in a majority of breast carcinomas (Huang *et al*., 2006). Findings of similar expression behavior were seen in a Type 2b (differentiated non-keratinizing) NPC cell line (Sim *et al*., 2018). Such observation remains to be identified in other types of NPC cells. In this paper we explored the association of *eL13* and *eL14* with NPC by assessing differential expression patterns between NPC and non-malignant nasopharyngeal epithelial cell lines.

MATERIALS & METHODS

Cell Culture

The NPC cell lines used in this study were HK1 (Huang *et al*., 1980), HONE1 (Glaser *et al*., 1989), TW01, TW04 (Lin *et al*., 1993), and SUNE1 (Teng, Ooka, Huang & Zeng, 1996). HONE1, SUNE1, and TW04 were from Type 2a NPC, HK1 was from Type 2b, and TW01 was from Type 1. The normal nasopharyngeal epithelial cell line, NP69 (Tsao *et al*., 2002) was the normal control. All NPC cell lines were cultured in RPMI-1640 media supplemented with 10% foetal calf serum (FCS), 10 U/ml penicillin and 10 µg/ml streptomycin. The NP69 cell line was cultured in keratinocyte serum-free media (DKSFM) supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2.

RNA Extraction and Reverse Transcription

Total cellular RNA was extracted from the cell cultures using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. Cells (in each culture flask) were rinsed with cold Phosphate Buffer Saline solution and treated with TRIzol reagent to be dislodged and homogenized. The homogenate was then transferred to 1.5 ml tube and mixed with chloroform (0.2 ml per 1 ml TRIzol). After incubation for 5 min, the mixture was centrifuged (10000 × g for 15 min at 4°C). The upper aqueous layer containing RNA was transferred to a new 1.5 ml tube and mixed with isopropanol. Following precipitation, the RNA was pelleted via centrifugation, washed with 75% ethanol, air-dried, and dissolved in nuclease-free water. Assessment of extracted RNAs included agarose gel electrophoresis (AGE) assay, and the optical density measurement at absorbance value of 260 and 280 nm using a UV spectrophotometer (Shimadzu, USA or Ultrospec 1100 pro, UK). RNAs of all cell lines were converted to cDNAs. First strand cDNA was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Promega, USA). Basically, 2 µg of RNA was reverse transcribed using MMLV in a reaction mixture containing 0.5 µg random primers, 1X reaction buffer, 40 mM dNTPs, 25 U Recombinant RNasin® Ribonuclease Inhibitor, and 200 U M-MLV RT. The reaction was incubated for 60 min at 37°C, and then at 70°C for 15 min. Synthesized complementary DNAs (cDNAs) were checked using AGE assay and kept at -20°C until use.

Polymerase Chain Reaction (PCR)

For each sample, the final PCR mixture (vol. of 25 µL) contained 1X Green Go Taq® Flexi Buffer, 1 mM $MgCl_2$, 0.2 mM dNTP mix, 0.625 U GoTaq® DNA Polymerase, 0.4 µM each of forward and reverse primers, and 0.25 µg of template cDNA. The cDNA template used was from five NPC cell lines (HK1, SUNE-1, HONE1, TW01, TW04) and one normal human nasopharyngeal cell line (NP 69). Thermal cycling conditions were initial denaturation at 95°C for 2 min; 30 cycles where each comprised denaturation at 95°C for 30 sec, annealing from 51 to 53.5°C (depending on primer type) for 30 sec, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. The sequences of primer pairs were 5'-CTTTCCGCTCGGCTG TTG-3' (forward) and 5'-GCCTTACGTCTGCGGATC-3' (reverse) with expected amplicon size of 163 bp for *eL13*; 5'-TTCTTCCTTCTCGCCTAACG-3' (forward) and 5'-CCTCCTAAC TTCAGCCTCAA-3' (reverse) with expected amplicon size of 773 bp for *eL14*; and 5'-AGATCATCAGAATGCCTC-3' (forward) and 5'-TACCAGGACATGAGCTTGAC-3' (reverse) with expected amplicon size of 511 bp for *GAPDH*. Duplicates were done for each test.

Data Acquisition and Analysis

Results of PCR assays were assessed using agarose gel electrophoresis (AGE) method. Band intensity was documented and measured using ImageQuant gel documentation system via the TotalLab Quant software. Subsequently, observed amplicon bands were gel extracted, purified, and sent to a DNA sequencing service provider for the acquisition of sequence data. After sequence verification, expression data (band intensity value) of each ribosomal protein gene were normalized by comparative assessment with associated expression data of the housekeeping gene, *GAPDH*. The derivation of normalized value was based on the formula below.

$$\text{Normalised value of test A} = \frac{\text{Average value of } \textit{GAPDH} \text{ band intensity}}{\text{Intensity value of } \textit{GAPDH} \text{ for test A}} \times \text{Intensity value of test A}$$

Statistical analysis of difference between detectable transcript expression was carried out using single factor ANOVA test. Significance (if any) of *eL14* differential expression among the NPC cell lines (TW01, TW04, and HONE-1) was taken at $p < 0.05$.

RESULTS

Following AGE analysis, bands representing amplicons of RP genes can only be detected for *eL14*. However, this is only observable for samples from TW01, TW04, and HONE-1 in Replicate Test 1; and TW01, and HONE-1 in Replicate Test 2 (Figure 1). The consistency of PCR assays for *eL14* in both sets of replicates was only relevant in TW01 and HONE-1 cell lines. There are no bands detected for *eL13* in all the cell lines studied. Bands for *GAPDH* were clearly detected in all cell lines studied. Observed bands for *eL14* and *GAPDH* were gel extracted, purified and sent for sequence analysis. The DNA sequences obtained verified that the band within the vicinity of 750 bp is the bone fide amplicon for *eL14* gene. Likewise, sequences for the band in the region of 500 bp verifies it to be the *GAPDH* gene. The validity of these amplicons allowed us to proceed further with extraction of band intensity values.

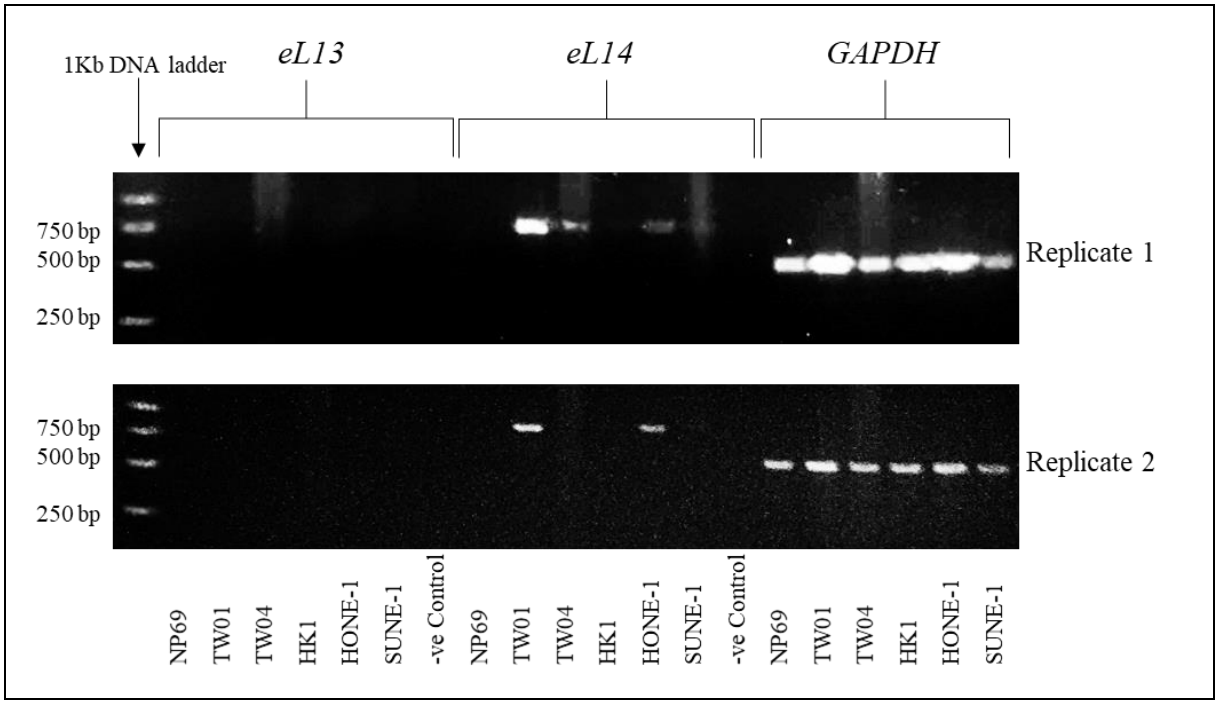

Figure 1. AGE analysis results of PCR amplicons for *eL13*, *eL14*, and *GAPDH* expressed transcripts in the NPC and normal control cell lines.

Raw band intensity values were only extractable for *eL14* amplicons in samples from TW01, TW04, and HONE-1 in Replicate Test 1; and TW01, and HONE-1 in Replicate Test 2 (Table 1). No values can be procured from *eL13* in all the cell lines studied. The band intensity values for the amplicons of the housekeeping gene, *GAPDH* were extracted from all the cell lines studied. Following the process of data adjustment of *eL14* against *GAPDH* values, normalized intensity data was derived for samples from TW01, TW04, and HONE-1 in Replicate Test 1;

and TW01, and HONE-1 in Replicate Test 2 (Table 1). From the normalized data, average value and standard deviation information were obtained also. A bar chart of the normalized data for *eL14* expression in all the cell lines was plotted (Figure 2). This indicated a general trend of *eL14* over-expression in TW01, TW04, and HONE-1 relative to the normal control cell line, NP69 (Figure 2). Nevertheless, based on assessment of the error bars alone, it can be inferred that only the expression in TW01 cell line is markedly different from the normal control (NP69).

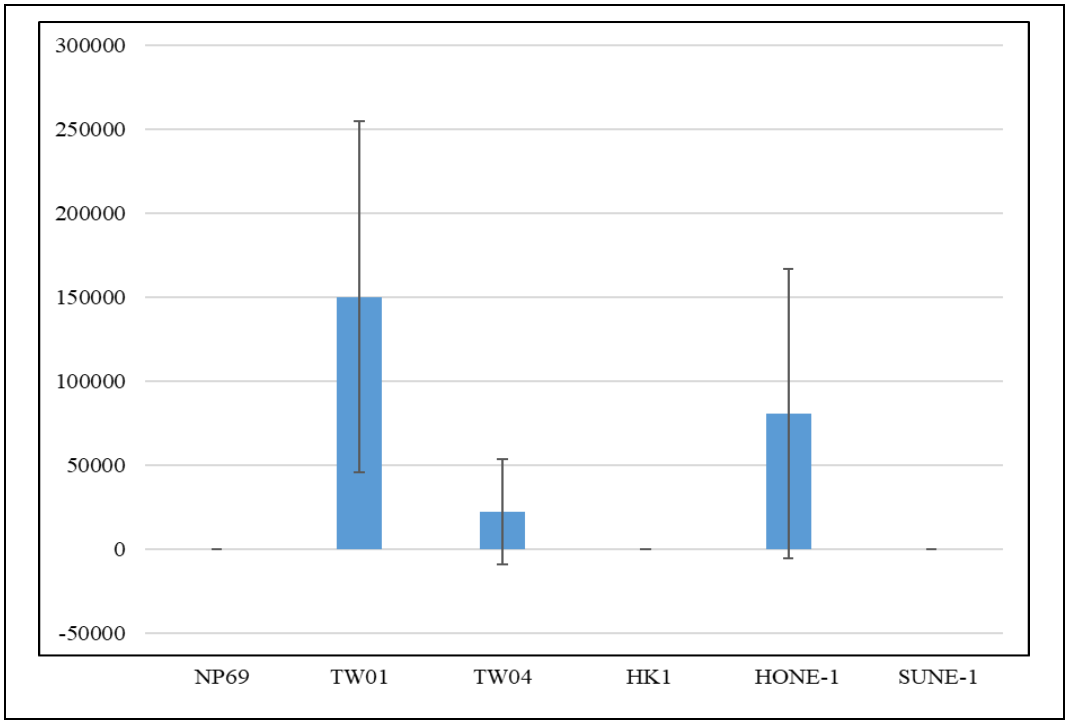

Figure 2. Bar chart of normalized band intensity values for *eL14* amplicons in the 5 NPC cell lines and one normal cell line studied. The y-axis and x-axis represent band intensity values and type of cell lines respectively. Error bars are standard deviation value of the average intensity data.

A single factor ANOVA test was carried out on the normalized data for *eL14* in samples TW01, TWO4, and HONE-1, as shown in Table 2. This statistical analysis reveals no significant difference in expression levels among the three NPC cell lines ($p = 0.397$, Table 2). Since *eL14* levels in TW04 and HONE-1 are not markedly different from NP69 (normal control) (Figure 2), and that statistical analysis cannot prove that its level in the three cell lines (TW01, TWO4, and HONE-1) is significantly different, it should be deduced that the observed general over-expression trend (Figures 1 and 2) cannot be not conclusively defined.

DISCUSSION

Our findings cannot demonstrate a clear association of *eL14* with NPC tumorigenesis. Firstly, we could not prove conclusively that it is significantly differentially expressed in NPC cell lines compared to a normal control, albeit the observation of a general over-expression in two of the NPC cell lines. In our previous study (Sim *et al*., 2018), *eL14* was shown to be significantly down-regulated in HK1 cell line (Type 2a-NPC) but in this report, this was not the case. In fact, its expression was not detected at all in HK1 cells. Strangely, it was also not detected in the normal or non-malignant cell line, NP69. Its expression, was however, detected in another Type 2a-NPC cell lines, HONE-1. The expression level is not remarkably differential in this cell line. The only markedly different (over-expression) pattern was observed for TW01. This is the first report that reveals an abundance of *eL14* expressed transcript in a Type 1 NPC category cell. Nonetheless, it is now necessary to reproduce similar exploration of *eL14* expression in TW01 cells and/or investigate its activity in Type 1 NPC tissues in order to have a more conclusive inference.

Table 1. Raw and normalized intensity values of amplicons observed in the AGE analysis of *eL14* in the 5 NPC cell lines and one normal control cell line studied.

Cell lines	*eL14* band intensities value for Replicate 1				*eL14* band intensities value for Replicate 2				Average nomalized	Error diff
	Raw	GAPDH	Average GAPDH	Normalized	Raw	GAPDH	Average GAPDH	Normalized		
NP69	0	103053	121756	0	0	340144	395599	0	0	0
TW01	110367	176572		76104	289902	511475		224224	150164	104737
TW04	36156	99924		44056	0	328327		0	22028	31152
HK1	0	147687		0	0	445450		0	0	0
HONE-1	22147	135855		19849	164653	459855		141637	80743	86118
SUNE-1	0	67446		0		288344		0	0	0

Table 2. Single Factor ANOVA test result of *eL14* expression data in the three NPC cell lines with observable amplicon bands.

Summary				
Cell lines	**No. of replicate**	**Sum**	**Average**	**Variance**
TW01	2	300328.2	150164.1	1.1E+10
TW04	2	44055.64	22027.82	9.7E+08
HONE-1	2	161485.9	80742.97	7.42E+09

ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	***p*-value**	**F crit**
Between cell lines	1.65E+10	2	8.23E+09	1.27532	**0.397345**	9.552094
Within cell lines	1.94E+10	3	6.45E+09			
Total	3.58E+10	5				

It is clear that our findings herein further obfuscate a pivotal hypothesis on the unequivocal association of RP genes (specifically, *eL14*) to the context of NPC carcinogenesis. However, this confusing scenario of their inconsistent expression behaviors between studies is not unprecedented. In 2008, we found *eS26* and *eS27* to be down-regulated in NPC tissues relative to normal samples (Sim *et al*., 2008), but only to conclude that these two RPs have no association with NPC in a later study (Ma *et al*., 2012). Similarly, when we reported the under-expression of *eL27*, *eL41* and *eL43* in NPC cell lines (Sim *et al*., 2010b), the observation was subsequently refuted in a later study by us (Sim *et al*., 2016), whereby these three RP genes were found to be over-expressed in NPC situation instead. Such discrepancy in findings between studies implies the complexity and subtlety of RP's involvement in NPC tumorigenesis and malignancy. As such, functional significance of RPs in NPC carcinogenesis remains a potent area for deeper molecular research.

The RP gene, *eL13* has never been studied in the context of NPC, and this paper is the first to explore its association with the cancer. We found no detectable expression of its transcripts in any of the cell line studied, including the normal/non-malignant NP69 cells. To infer its inactiveness in neoplastic cells/tissues may be a premature conjecture. In fact, its ubiquitous state of expression in clear-cell renal carcinoma is evident regardless of clinico-pathological presentation (Wierzbicki *et al*., 2014). Perhaps, this RP gene is specifically not active in the cells from the epithelium of the nasopharynx region, and is also not dysregulated when cells in that region are in a neoplastic situation. To suggest that its non-association with nasopharyngeal organogenesis or tumorigenesis requires further research, particularly in the interrogation of its expression in NPC tissues.

On overall, our data is limited by some inconsistency, particularly in the result of *eL14* in TW04. Therefore, an experimental repeat of *eL14* expression in this cell line is necessary. In fact, more replicates should be applied for all cell lines should this study be carried further. An additional caveat to our work here is the limitation of our methodology. Our use of the conventional RT-PCR technique should suggest a cautionary interpretation of our results. This technique lacks accuracy as far as measurement of real time gene amplification is concerned. Hence, it is only useful for the relative assessment of expression level based on end-point quantification of amplicon (Walker, Worst & Vrana, 2003). It also lacks sensitivity in the detection of trace level expressed transcripts. Future studies on *eL13* and/or *eL14* in NPC cells or tissues will have to consider the more accurate and sensitive technique of quantitative PCR (or Real Time PCR) method.

CONCLUSION

Expression at transcript level of ribosomal protein gene, *eL14* was detected in three nasopharyngeal carcinoma cell lines, namely TW01 (Type 1-NPC), and HONE-1 (Type-2a-NPC). The highest level was observed for TW01 cell line. However, the expression pattern was not significantly differential among the three cell lines, and not distinctly diverse to non-malignant scenarios. Besides this, the expression of the ribosomal protein gene, *eL13* was not detected in all the cell lines studied. Taken together, our results cannot provide a conclusive finding on any evident association between *eL13* or *eL14* and tumorigenesis or malignancy of the human nasopharynx. Instead, we suggest a vastly tenuous nature of relationship between ribosomal proteins and oncogenesis of NPC.

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