

First Record of Identification of *RPMS1* Gene Variations in Vietnamese Nasopharyngeal Carcinoma Patients

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Abstract

Epstein-Barr Virus (EBV) infection and EBV genes' variation are considered as the etiological factors contributed to Nasopharyngeal Carcinoma (NPC). In the latent EBV stage, not only the latent membrane proteins but also *RPMS1* expression has been confirmed in all EBV-associated tumors. In Vietnam, an Asian country with the high incidence, mortality rate of NPC, had limit research on the *RMPS1* gene variation. Therefore, the objects of current study were to identify the pattern of *RMPS1* variations in Vietnamese NPC patients for its value further applied in NPC patients. In this study, thirty NPC biopsy samples and thirty non-cancerous swab specimens were collected from local patients, analyzed by PCR, sequencing and compared to previous B95-8 sequence. As the results, the strongly association between the detection of *RPMS1* gene and NPC incidence in Vietnamese NPC patients was determined. Additionally, the *RMPS1* gene variants, including wild-type, *RMPS1-B*, *RMPS1-C*, *RMPS1-C**, were identified. Among them, *RMPS1-C/C** was preferential in Vietnamese nasopharyngeal cancer. In conclusion, those data is the first dataset on the polymorphism in the *RPMS1* gene in Vietnamese NPC patients, and could be utilized as a promising biomarker for prognosis, diagnosis and therapy for NPC based on the EBV gene variations.

Keywords: Nasopharyngeal Carcinoma, *RPMS1*, Variations

1. Introduction

The epidemiology of Naso-Pharyngeal Carcinoma (NPC), a prevalent malignant tumor of nasopharynx, has remarkably characterized by its distinctive geographic and ethnic contribution, gravitating toward Southern Asia. Vietnam has one of the highest incidence rates of NPC in worldwide^{1,2}. According to statistics of Globocan (2012), the high prevalence of NPC cases was observed

in reached to 4,931 cases (ASR = 5.4/100,000) and deaths was 2,885 cases (ASR = 3.3/100,000) in Vietnamese population³. For the past few years, many studies have been demonstrated that the major etiological factors proposed for NPC pathogenesis, including Epstein-Barr virus (EBV) infection, genetics/or epigenetic changes and environmental factor⁴⁻⁸. In the latent EBV stage, additionally to the latent membrane proteins (LMP-1, LMP-2), and small noncoding RNAs (EBERs), as

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the member of the BamHI-A Rightward Transcripts (BARTs) family, *RPMS1* expression has been confirmed in all EBV-associated tumors⁹⁻¹³. A number of studies have reported attempts to identify the NPC associated EBV genes' variations, including EBV Nuclear Antigens (EBNAs), Latent Membrane Proteins (LMPs) and EBV-encoded small nuclear RNAs (EBERs), have been shown to be associated with NPC pathogenesis¹⁴⁻¹⁷. However, few studies have investigated the single nucleotide polymorphisms (SNPs) of *RPMS1* gene, as the reported, only one SNP site (locus: 155391 G > A, named G155391A) is identified and significantly associated with NPC incidence^{16,18}. They found that the *RPMS1* variation (G155391A) functionally relevant to regulating the *RPMS1* protein stability and over expressed *in vitro*¹⁶.

Vietnam, located in Southern Asia, is well known as the high incidence and mortality rate of nasopharyngeal carcinoma in the world. According to EBV infection, the rate of EBV positive in NPC patients is high¹⁷. However, limited studies were carried on the genotyping of EBV as well as the variations of EBV genes. In previous studies, we established the protocol for genotyping of EBNA-1 subtype from nasopharyngeal biopsy samples collected from Vietnamese nasopharyngeal carcinoma patients, and the results suggested the V-val subtype is the preferentially subtype associated with nasopharyngeal carcinoma. To further identify the EBV variations linked to NPC risks, we conducted the analysis on the *RPMS1* gene, a member of *BARTs* family, in the comparison to the prototype B95-B strain, starting from NPC biopsy samples and healthy controls in the Vietnamese population. Therefore, our objective was to develop the simple method to identify the *RPMS1* gene's polymorphisms at Vietnamese population.

2. Materials and Methods

2.1 Ethics Statement

Institutional Ethics Board approval, the decision number of the permission from Ethical committee: 516/BVCR-

HDDD, was obtained from the Medical Ethics Committee of the Cho Ray Hospital, Ho Chi Minh City, Vietnam. All the biopsy samples used in current study were collected from participants, who agreed and signed on the consent forms.

2.2 Samples Collection, and DNA Isolation

Thirty nasopharyngeal carcinoma biopsy samples were collected from local patients, in Cho Ray Hospital, Ho Chi Minh City, Vietnam. The entire sample was submitted to the histopathological diagnosis center to confirm the NPC. Notably, all of those biopsies were collected from patients, who were obeyed to ethical approval for study human samples, and patients agreed with purpose of the study. In addition, thirty nasopharyngeal swab samples, which were collected from healthy donors, used as negative-nasopharyngeal carcinoma control.

For DNA isolation, biopsies were lysed in lysis buffer, containing 10 mM Tris-HCl pH=8, 10 mM EDTA, 150 mM NaCl, 2% SDS, and 0.1mg/ml Proteinase K, incubated in 56°C, overnight. Total of genomic DNA of clinical samples were extracted by using Phenol/Chloroform solution, pH = 8. The purification of DNA was done by Ethanol solution 99%. The quality and purity of DNA isolates were measured by the evaluation of A_{260}/A_{280} proportion. The DNA solution was stored in Tris-EDTA 0.5M, at -20°C for further assays.

2.3 PCR-sequencing of *RPMS1* Gene

Multiplex PCR assay was applied for the amplification of *RPMS1* gene and beta-actin gene, which served as the internal control. The sequence and position of primers were shown in (Table 1).

The PCR assay was performed in a total of 15 μ L, containing 100 ng genomic DNA templates, 0.25 μ M each primer, and 7.5 μ L MyTaqTM Mix (Bioline, Cat No. BIO-25041). PCR amplification was performed with an initial denaturation at 94°C for 5 minutes; followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds; and

Table 1. Primers' sequences used in current study

| Target genes | Primer | Sequence (5' - 3') | Reference |
|-------------------|---------|-------------------------|-----------|
| <i>RPMS1</i> | RPMS1-F | GCTGGGTTGATGCTGTAGATG | 16 |
| | RPMS1-R | AGGGTCTGGACGTGGAGTTTG | |
| <i>Beta-actin</i> | Beta-F | ATCATGTTTGAGACCTTCAACAC | 19 |
| | Beta-R | CATCTCTGCTCGAAGTCCAG | |

elongation step at 72°C for 10 minutes. The PCR products were visualized and analyzed by electrophoresis through stained with Ethidium bromide, and 2.0% agarose gel. For *RPMS1* gene sequencing, 30 µL products were sent to Nam Khoa Biotek Co. Ltd. (Vietnam) for directly sequencing in both directions with primer of *RPMS1*.

2.4 Determination and Analysis of *RPMS1* Variations

The results of *RPMS1* sequencing was read by Chromas 2.6.4 (Technelysium) and checked for sequencing homology by using BLAST (NCBI). For determination of *RPMS1* variations, the sequencing of *RPMS1* of current study were compared to the B95-8 strain (Genbank accession number: NC_007605; Gene *RPMS1*'s Location 138352..160531). The alignment between data sequence and B95-8 strain sequence were analyzed by using BioEdit sequence alignment editor.

3. Results

3.1 Association between *RPMS1* and NPC Incidence

The frequency of *RPMS1* gene in NPC biopsy samples and healthy samples were detected by PCR method. The PCR product sizes, 319-bps and 733-bps bands, were different which is easy to be distinguished on electrophoresis results, shown in (Figure 1). The human beta-actin PCR product of 319 bps gave the indication on the good quality of the

extracted DNA and PCR assay. Overall, the frequency of *RPMS1* in NPC samples was 66.67% (20 of 30 samples). In non-NPC swab samples, no *RPMS1*-positive case was detected. Based on the statistical analysis, the presence of *RPMS1* was found to be significantly associated with NPC ($p < 0.05$). Additionally, the 733-bp product, which indicated the presence of *RPMS1*, was determined by Sanger sequencing. The signals of peaks in PCR product sequencing were good for nucleotide reading (Figure 2). According to BLAST results, candidate gene's sequence was similar to Human gammaherpes virus 4 (Accession number: MH144220) within the Total score = 1216, ident = 99.85%, E-value = 0.0.

3.2 Identification of *RPMS1* Gene Variations: *RPMS1*-C Variation was Preferential in Vietnamese Nasopharyngeal Cancer

RPMS1 gene was successfully amplified, observed in a distinct band with 733 bps, shown in Figure 1 and sequenced in 20 of 30 NPC samples. The nucleotide variations were determined by mapping and comparing to the *RPMS1* gene of wild-type EBV genome (GenBank Accession No. NC_007605: location 138352..160531). As the results, nice NPC samples (counting for 45.0%) were similar to the wild-type EBV's *RPMS1* gene. The polymorphisms point G155391A, classified as *RPMS1*-C, was discovered in 10 NPC samples (counting for 50.0%). Of these, 1 NPC sample differentially distributed substitution G155391A and one silent polymorphism

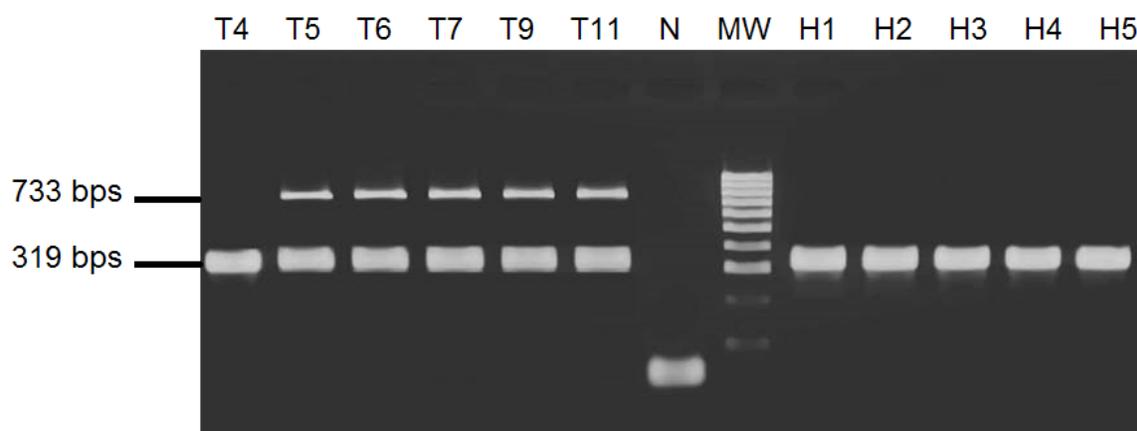


Figure 1. Electrophoresis of Nested-PCR products of representative clinical samples: T4, T5, T6, T7, T11: clinical biopsy samples; H1, H2, H3, H4, H5: Healthy (non-NPC) swab samples; N: negative control; MW: molecular weight 100 bps.

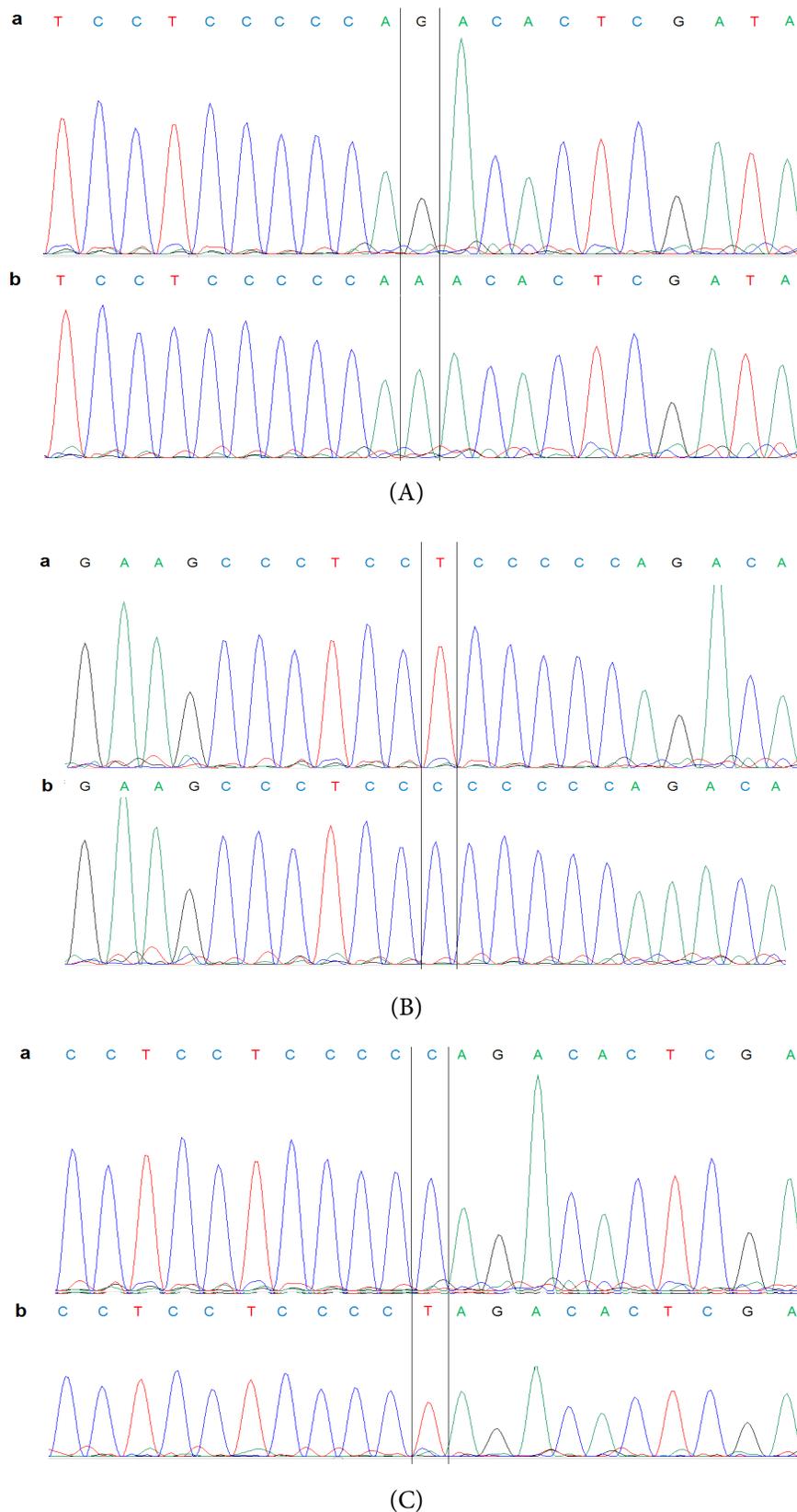


Figure 2. Validation of the *RPMS1* single nucleotide polymorphism (A) G155391A; (B) T155384C; (C) C155389T in representative NPC samples by Sanger sequencing. (a) wild type *RPMS1* gene (NC_007605); (b) representative SNPs identified samples.

site as T155384C, classified as RPMS1-C* (Figure 2A, B). Moreover, only one sample, counting for 5.00%, within the substitution C155389T, classified as RPMS1-B, was determined (Figure 2C) (Table 2). Therefore, RPMS1-C variation was preferential in Vietnamese nasopharyngeal cancer.

All the nucleotide variations of sequencing, encoded the amino acid residue 48, 50, 51 of *RPMS1* gene, of 20 cases were determined by comparing with the B95-8. As the results, three patterns of RPMS1, including RPMS1-B, RPMS1-C, RPMS1-C*, and wild type (Wt), were determined (Table 2). In detail, Wt (amino acid at 51 (GAC:Asp), representatively reported by T6), RPMS1-B (the amino acid change was at residue 50 (CCA:Pro>CTA:Leu), representatively T22), RPMS1-C and RPMS1-C* (the amino acid change was at residue 51 (GAC:Asp>AAC:Asn), representatively reported by T5), were similar to previous studies. The RPMS1-C and RPMS1-C* shared the same pattern, in spite of the T155384C occurred in RPMS1.

4. Discussion

In current study, we successfully amplified *RPMS1* gene from NPC biopsy samples by multiplex-PCR with the internal control – *beta actin*. Notably, to our knowledge,

the current study is the initial study, which recorded the determination of *RPMS1* polymorphisms in total of 20 RPMS1-positive samples collected from Vietnamese NPC patients. As the results, T155384C variant was identified as the novel variant of RPMS1 variations from Vietnamese nasopharyngeal carcinoma patients. However, this variant did not effected the primary structure of translated protein, due to the silence substitution (CCT: Pro>CCC:Pro). Therefore, in summary, three patterns, RPMS1-B, RPMS1-C/C*, and Wt, were identified. In previous studies, four RPMS1 patterns were determined and clustered as RPMS1-A, RPMS1-B, RPMS1-C and RPMS1-D (compared to wild type EBV genome). A RPMS1-C pattern variation was represented by the *RPMS1* SNP G155391A (amino acid residue GAC:Asp>AAC:Asn). This variation has been reported to be associated with a high risk of NPC¹⁶. According to Feng *et al.*, they suspected that the variation of G155391A from Guanine to Adenine, leading to the amino acid change from Aspartic acid (Asp) to Asparagine (Asn), might related to the expression of *RPMS1*¹⁶. In detail, the variations of G155391A are functionally relevant to the stability of RPMS1, which is in a longer half life of RPMS1 protein and may exhibit stronger carcinogenesis potential. Additionally, the oncogenic RPMS1 has shown to promote the cell differentiation and proliferation, thus,

Table 2. Nucleotide and amino acid substitution in *RPMS1* gene compared to wild type B95-8 sequence

| Patterns | Nucleotide Position | 155382 | 155383 | 155384 | 155388 | 155389 | 155390 | 155391 | 155392 | 155393 | Frequency |
|----------|---------------------|-----------------------|--------|--------|--------|--------|--------|--------|--------|--------|-----------|
| | | Wild type (NC_007605) | | | Pro | | | Asp | | | |
| Codon | | 48 | | | 50 | | | 51 | | | |
| RPMS1-B | T22 | . | . | . | . | T | . | . | . | . | 5.0% |
| | | * | | | Leu | | | * | | | |
| RPMS1-C | T5 | . | . | . | . | . | . | A | . | . | 50.0% |
| | | * | | | * | | | Asn | | | |
| RPMS1-C* | T9 | . | . | C | . | . | . | A | . | . | |
| | | + | | | * | | | Asn | | | |
| Wt | T6 | . | . | . | . | . | . | . | . | . | 45.0% |
| | | * | | | * | | | * | | | |

Note: The top four rows were corresponding to the nucleotide, amino acid position, nucleotide and amino acid of B95-8 prototype sequence. In each row, the upper character denoted the nucleotide, which differed to the B95-8 prototype sequence. Conversely, the dot (.) denoted the same nucleotide compared to the referent sequence. The below characters indicated the amino acid written in three letter code that differed to the referent sequence. On the contrary, the “*” character indicated the unchanged amino acid, and the “+” character indicated the silent amino acid changes.

may exhibit stronger carcinogenesis potential leading to NPC^{16,20}. In current study, among identified pattern of *RPMS1*, *RPMS1-C/C**, counting for 50%, was preferential in Vietnamese nasopharyngeal cancer. Therefore, it could be partly explained that the *RPMS1-C/C** may contribute the high risk of NPC in Vietnamese population.

5. Conclusion

In summary, the significant association between the detection of *RPMS1* gene and NPC incidence in Vietnamese NPC patients was determined. Of these, the frequency of *RPMS1* gene was 66.67% in NPC clinical biopsy samples. Meanwhile, no positive case was detected in non-cancerous samples. Additionally, we successfully established the strategy to determine the *RPMS1* gene variations in Vietnamese nasopharyngeal patients. Three *RPMS1* gene variation patterns were identified in Vietnamese NPC patients. Among them, *RPMS1-C* was the preferred type in Vietnamese NPC patients. This approach may facilitate in identification of individuals who are at the high risk for NPC development, as well as further develop promising biomarker for prognosis, diagnosis and therapy for NPC based on the EBV gene variations.

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7. Ethics Approval and Consent to Participate

All patients signed inform consent before entering into the study. No study drug or procedure was applied. This is an observational study.

8. Conflict of Interest

The authors declared that they have no competing interests.

9. References

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