

NOVEL PATTERNS OF THE EPSTEIN-BARR NUCLEAR ANTIGEN (EBNA-1) V-VAL SUBTYPE IN EBV-ASSOCIATED NASOPHARYNGEAL CARCINOMA FROM VIETNAM

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ABSTRACT

The Epstein-Barr nuclear antigen 1 (*EBNA-1*) gene, plays a key role in viral infection, immortalization, viral genome replication, transcription and maintenance, and is the frequently detected gene, protein in both latent and lytic stage of Epstein-Barr virus (EBV). Based on the amino acid at position 487, EBNA-1 was classified into five subtypes, including P-Ala, P-Thr, V-Val, V-Pro and V-Leu. In Vietnam, an Asian country with a high incidence, mortality rates of nasopharyngeal carcinoma (NPC), had limited research on the EBNA-1 variation. Therefore, the aim of the current study was to identify the pattern of the EBNA-1 V-Val subtype in Vietnamese NPC patients, for its value further applied in NPC patients. Fifty-eight NPC biopsy samples were collected from local patients, analyzed by nested-polymerase chain reaction (nested-PCR), sequencing and compared to a previous B95-8 prototype sequence. Four EBNA-1 subtypes, including V-Val (35/44, 79.55%), P-Ala (2/44, 4.55%), P-Thr (5/44, 11.36%), and V-Leu (2/44, 4.55%), were observed in 44/58 samples. The sequences of the V-Val subtype were compared to the B95-8 prototype, resulting in five patterns, contained seven consensus changes, including five amino acid changes at positions 487, 499, 502, 524, 594, and two silent changes at residues 520 and 553. Of these, four of five, patterns were identified as novel patterns of the V-Val subtype, showing the different changes of amino acids at positions 492, 528, 529, 553, 585 and 588, by comparison with previous studies of V-Val EBNA-1. Those data suggested the profile of vari-

ation patterns of the *EBNA-1* gene, related to geographic distribution, in Vietnamese NPC patients.

Keywords: Epstein-Barr nuclear antigen (EBNA-1); Epstein-Barr virus (EBV); V-val; Variation.

INTRODUCTION

Epstein-Barr virus (EBV), a ubiquitous human gamma herpes virus, discovered in 1964 by Epstein and Barr, taxonomically identified as human herpes virus 4, is an oncogenic virus causing many EBV-associated human malignancies, including Hodgkin's disease, gastric carcinoma, B-cell lymphomas, and nasopharyngeal carcinoma (NPC) [1-5]. Following the infection of EBV, it remains in two stages: latency, lytic or productive phase of the life cycle of EBV [6]. Many processes of the viral cycle was dependent on the Epstein-Barr nuclear antigen (EBNA-1). Epstein-Barr nuclear antigen 1, a 641 amino acid protein, is the only consistently detected viral protein in many human EBV-associated malignancies [3,7,8]. Epstein-Barr nuclear antigen 1 is essential for EBV immortalization, the maintenance, replication and transcription of EBV genome, segregate EBV episome at mitosis [4,9]. A high frequency of sequence substitutions was identified in the carboxyl terminal (residues 325-641) of EBNA-1, containing many functional domains: the dimerization domain (residues 501-532 and 554-598), DNA binding domain (residues 459-487) and the transactivation domain (residues 450-641) [8,10-12]. Sequence variants occurring at the C-terminal of EBNA-1 may affect the functions of EBNA-1 protein, and consequently, affect DNA replication, transcription, or oncogenic potential EBV [9,13]. The sequence substitutions were frequently identified between residue 487 and 583 [14]. The two main mutant groups were identified and termed as prototype strains (similar to the prototype isolated from B95 cell line) and variant strains, which differed by 15 amino acids [14]. The further subtype identification

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was based on the amino acid at position 487 [14,15]. The EBV isolates could be divided into five subtypes: there are two prototype strains (P-strain) with either an alanine residue (termed as P-Ala), or a threonine residue (termed as P-Thr) at position 487, and three variant strains (V-strain) with either a leucine residue (termed as V-Leu), a valine residue (termed as V-Val) or a proline residue (termed as V-Pro) at this position [14,15]. These EBNA-1 subtypes display an association with geographical and human disease distribution. In these, V-Val is more frequent in NPC and almost detected in Chinese population, and preferentially exists in biopsies of NPC [15,16].

Vietnam, located in Southern Asia, is well known for the high incidence and mortality rate of NPC in the world [17]. However, most studies on EBNA-1 subtype classification were limited to NPC in the Chinese, not in Vietnamese population. In our previous study, we established the protocol for genotyping of the EBNA-1 subtype from of nasopharyngeal biopsy samples collected from Vietnamese NPC patients, and the results suggested that the V-Val subtype is the preferentially subtype associated with NPC [18]. In this current study, in order to explore the characteristics of the V-Val subtype in the Vietnamese population, we analyzed the polymorphisms of the *EBNA-1* gene in EBV-associated NPC in the comparison to the prototype B95-8 strain.

MATERIALS AND METHODS

Ethics Statement. The Institutional Ethics Board approval, the decision number of the permission from Ethics 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 all the consent forms.

Sample Collection and DNA Isolation. Fifty-eight NPC biopsy samples were collected from local patients at the Cho Ray Hospital, Ho Chi Minh City, Vietnam. All the sample were submitted to the histopathological diagnosis center to confirm the NPC diagnosis. Notably, all biopsies were collected from patients, who obeyed the ethical approval for study of human samples, and patients who agreed with the purpose of the study.

For DNA isolation, biopsies were lysed in lysis buffer, containing 10 mM Tris-HCl pH 8, 10 mM EDTA, 150 nM NaCl, 2.0% SDS, and 0.1 mg/mL Proteinase K, incubated overnight at 56 °C. Total of genomic DNA of clinical samples was extracted using a phenol/chloroform solution, pH 8. The purification of DNA was done by a 99.0% ethanol solution. 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.

The PCR Amplification of *EBNA-1* C-Terminal Domain of Clinical Samples and C-Terminal Domain Sequencing. Nested polymerase chain reaction (nested-PCR) was applied to amplify the C-terminal region of EBNA-1. The sequence and position of primers used in two stages of nested-PCR are shown in Table 1 and Figure 1, respectively. The EBNA-1-1 and EBNA-1-2 primers were used for stage 1 of nested-PCR, and EBNA-1-3 and EBNA-1-4 primers were used in stage 2 of nested-PCR. Both stages of nested-PCR were performed in a total of 15 µL, containing 100 ng genomic DNA template (stage 1) or 100 ng stage 1 PCR product (stage 2), 0.5 µM each primer, and 7.5 µL MyTaq™ Mix (Cat. No. BIO-25041; Bioline UK, London, UK).

Table 1. The sequence of primers used in two stages nested-PCR.

Stage	Primers	Sequences	Temperature
1	EBNA-1-1 EBNA-1-2	5'-TAG TCA GTC ATC ATC ATC CG-3' 5'-GGG ATT TAT TCT TTA GTG CG-3'	55 °C
2	EBNA-1-3 EBNA-1-4	5'-GCC ATT TTT CCA CCC TGT AG-3' 5'-ATT GAG GGC GTC TCC TAA CA-3'	60 °C

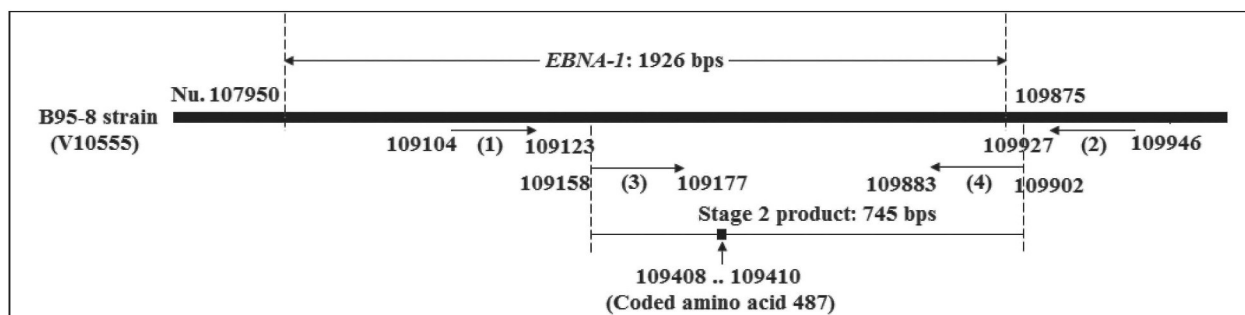


Figure 1. Schematic of the *EBNA-1* gene and position of primers aligned with B95-8 strain (V10555). Primer 1: EBNA-1-1; primer 2: EBNA-1-2; primer 3: EBNA-1-3 and primer 4: EBNA-1-4.

The PCR amplification was performed with an initial denaturation at 94 °C for 5 min.; followed by 45 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C (stage 1) or 65 °C (stage 2) for 30 seconds, extension at 72 °C for 30 seconds; and elongation step at 72 °C for 10 min. The nested-PCR products were visualized and analyzed by electrophoresis through stained with ethidium bromide, and 2.0% agarose gel. For EBNA-1 C-terminal sequencing, 30 µL products of the second stage of nested-PCR were sent to Nam Khoa Biotek Co. Ltd. (Ho Chi Minh City, Vietnam) for direct sequencing in both direction with primers EBNA-1-3 and EBNA-1-4.

Determination and Analysis of EBNA-1 V-Val Subtypes. The results of EBNA-1 C-terminal sequencing was read by Chromas 2.6.4 (Technelysium; <https://technelysium.com.au/wp/chromas/>) and checked for sequencing homology using BLAST [National Center for Biotechnology Information (NCBI); <https://www.ncbi.nlm.nih.gov/>]. For determination of EBNA-1 C-terminal subtypes, according to the study of Bhatia *et al.* [14] and Gutiérrez *et al.* [15], amino acid at position 487 was used as the signature residue for EBNA-1 subtypes. For the current study, the valine residue at position 487, identified as V-Val subtype, was enrolled into the analysis of V-Val characteristics of Vietnamese NPC patients. All the V-Val subtype sequence data were compared with the P-Ala B95-8 strain (Genbank accession #V10555). The alignment between data sequence and B95-8 strain sequence were analyzed using BioEdit (www.mbio.nesu.edu/BioEdit/bioedit.html) sequence alignment editor.

RESULTS

V-Val EBNA-1 Subtype was Preferential in Vietnamese Nasopharyngeal Cancer. The C-terminal domain of EBNA-1 was successfully amplified, observed in a dis-

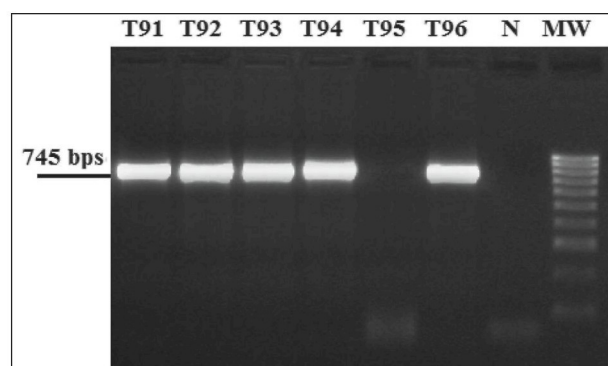


Figure 2. Electrophoresis of nested-PCR products of the represented NPC biopsy samples. T91, T92, T93, T94, T95, T96: clinical biopsy samples; N: negative control; MW: molecular weight 100 bp.

tinct band with 745 bp (shown in Figure 2) and sequenced in 44 of 58 clinical samples, accounting for 75.86%. The nucleotide variations were identified by comparison with the B95-8 P-Ala sequence. The sequencing results showed that P-Ala, P-Thr, V-Val and V-Leu were identified based on the amino acid at position 487 (Figure 3). The proportion of P-Ala, P-Thr, V-Val and V-Leu were two of 44 cases (accounting for 4.55%), five of 44 cases (accounting for 11.36%), 35 of 44 cases (accounting for 79.55%) and two of 44 cases (accounting for 4.55%), respectively. Thus, it was concluded that the V-Val EBNA-1 subtype was the common subtype, and V-Pro was not detected in Vietnamese nasopharyngeal cancer.

Characteristic of V-Val Sequence patterns of EBNA-1 in Vietnamese Nasopharyngeal Cancer. All the nucleotide variants of sequence, encoded the amino acid residue 487 of the EBNA-1 C-terminal domain, 35 cases of V-Val EBNA-1 were determined by comparison with the B95-8 sequence. As a result, five patterns of V-Val subtype of EBNA-1 C-terminal domain, coded pattern 1 to pattern 5, were identified (Table 2). All of those five patterns contained seven consensus changes, including five amino acid changes and two silent changes (Figure 4). The five amino acid changes were at residue 487 (Ala→Val; GCT>GTT), 499 (Asp→Glu; GAC>GAG), 502 (Thr→Asn; ACT>AAT), 524 (Thr→Ile; ACT>ATT), 594 (Arg→Lys; AGG>AAG). The two silent amino acid changes were at residue 520 (Leu→Leu; CTA>CTC) and 553 (Pro→Pro; CCG>CCA). In the V-Val subtype, pattern isolates showed two or more additional changes except for the consensus sequence changes (Figure 5). There were pattern 1: 17 of 35 isolates (accounting for 48.57%, represented by T5) with additional amino changes at residue 528 (Ile→Val; ATT>GTT), 533 (Leu→Ile; CTT>ATT); pattern 2: 14 of 35 isolates (accounting for 40.00%, represented by T6) with additional substitutions at residue 528 (Ile→Val; ATT>GTT), 533 (Leu→Ile; CTT>ATT), 585 (Thr→Ile; ACA>ATA); pattern 3: 2 of 35 isolates (accounting for 5.71%, represented by T77) with additional changes at residue 529 (Pro→Gln; CCA>CAA), 585 (Thr→Ile; ACA>ATA), 588 (Ala→Pro; GCT>CCT); pattern 4: one of 35 isolates (accounting for 2.86%, represented by T70) with additional changes at amino acid 528 (Ile→Val; ATT>GTT), 533 (Leu→Ile; CTT>ATT), 585 (Thr→Ile; ACA>ATA), 588 (Ala→Pro; GCT>CCT); pattern 4: one of 35 isolates (accounting for 2.86%, represented by T75) with additional amino acid changes at position 492 (Ser→Cys; AGT>TGT), 528 (Ile→Val; ATT>GTT), 533 (Leu→Ile; CTT>ATT), 585 (Thr→Ile; ACA>ATA), 588 (Ala→Pro; GCT>CCT). Therefore, the common pattern of V-Val subtypes were pattern 1 (accounting for 48.57%) and pattern 2 (accounting for 40.00%).

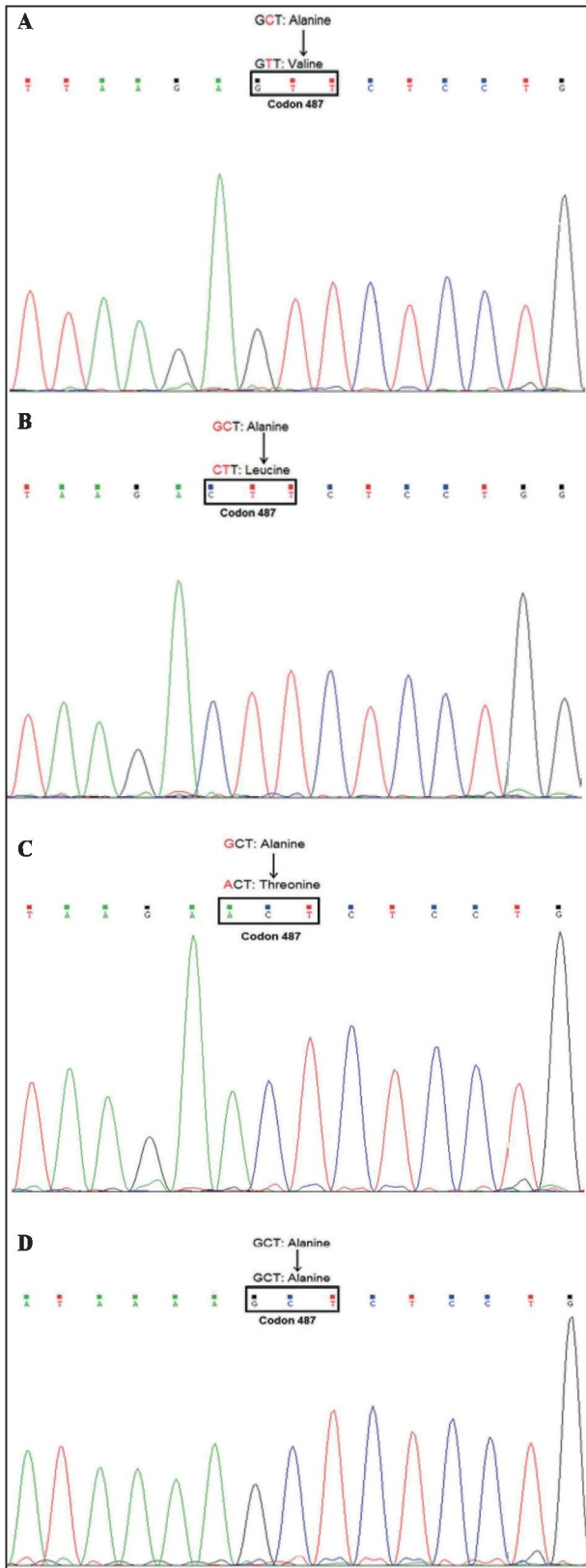


Figure 3. Represented sequence identification of EBNA-1 subtype based on the amino acid at position 487. A) T91: V-Val subtype; B) V-Leu subtype; C) P-Thr subtype; D) P-Ala subtype.

Novel Patterns of V-Val Subtype of the EBNA-1 C-Terminal Domain from Vietnamese Patients. The previous study of Wang *et al.* [19] reported that the common pattern of EBNA-1 C-terminal domain contained 12 consensus sequence changes, including 10 amino acid changes at positions 487, 499, 502, 524, 528, 533, 594, and two silent changes at residues 520 and 553. Additionally, those 12 consensus sequence changes were reported to be the same as the sequences of published V-Val subtype or its sub-variants at the sequenced part in previous studies [13,15,20-23]. Compared to our study, 10 amino acid at positions 487, 499, 502, 520, 524, 553 and 594 were the same as the GC4 (Table 3). The amino acid at positions 528 and 533 were different to the consensus amino acid of the GC4 sequence. Moreover, besides the consensus substitutions, which were reported in GC4, in our patterns of EBNA-1 variants, the amino acid at position 492 (Ser→Cys) by T75, 528 (without change: Ile); 529 (Pro→Gln); 553 (with change: Leu) by T77; 585 (Thr→Ile) by T6, T77, T70, T75; and 588 (Ala→Pro) by T77, T70, T75, were different to GC4 (Table 3). Therefore, it could be concluded that pattern 1 (represented by T5) was the same as the GC4. Pattern 2 (represented by T6), pattern 3 (represented by T77), pattern 4 (represented by T70), and pattern 5 (represented by T75) were different to GC4, thus, identified as the novel patterns of EBNA-1 C-terminal domain in Vietnamese NPC.

DISCUSSION

In the current study, we characterized the variations of the C-terminal domain of EBNA-1 sequences in a total of 44 EBNA-1-positive samples collected from Vietnamese nasopharyngeal cancer patients. To the best of our knowledge, this is the largest study of EBNA-1 sequence identification on EBV infection in Vietnamese cases on the high incidence and mortality rate of NPC in the world.

In general, four EBNA-1 subtypes were designed as V-Val, P-Ala, P-Thr and V-Leu. These findings were similar to the research of Sun *et al.* [9] and showed that four subtypes of EBNA-1, except for V-Pro, were observed. It was related it to the geographically-associated EBNA-1 subtype distribution. Of these, V-Val, accounting for 79.55%, was the predominant subtype in Vietnam, an Asian country, which is similar to that of previous studies in Asian countries, such as Hong Kong; Chinese population but different from Western countries [9,15,16,20,23]. The V-Val subtype was reported as the isolate-induced higher transcriptional activity than those of the B95-8 subtype [13]. Additionally, in the current study, the nucleotide variations were analyzed between the nucleotide regions from 109408 to 109731, encoding the amino acid region from 487 to 594. As a result, most of the substitu-

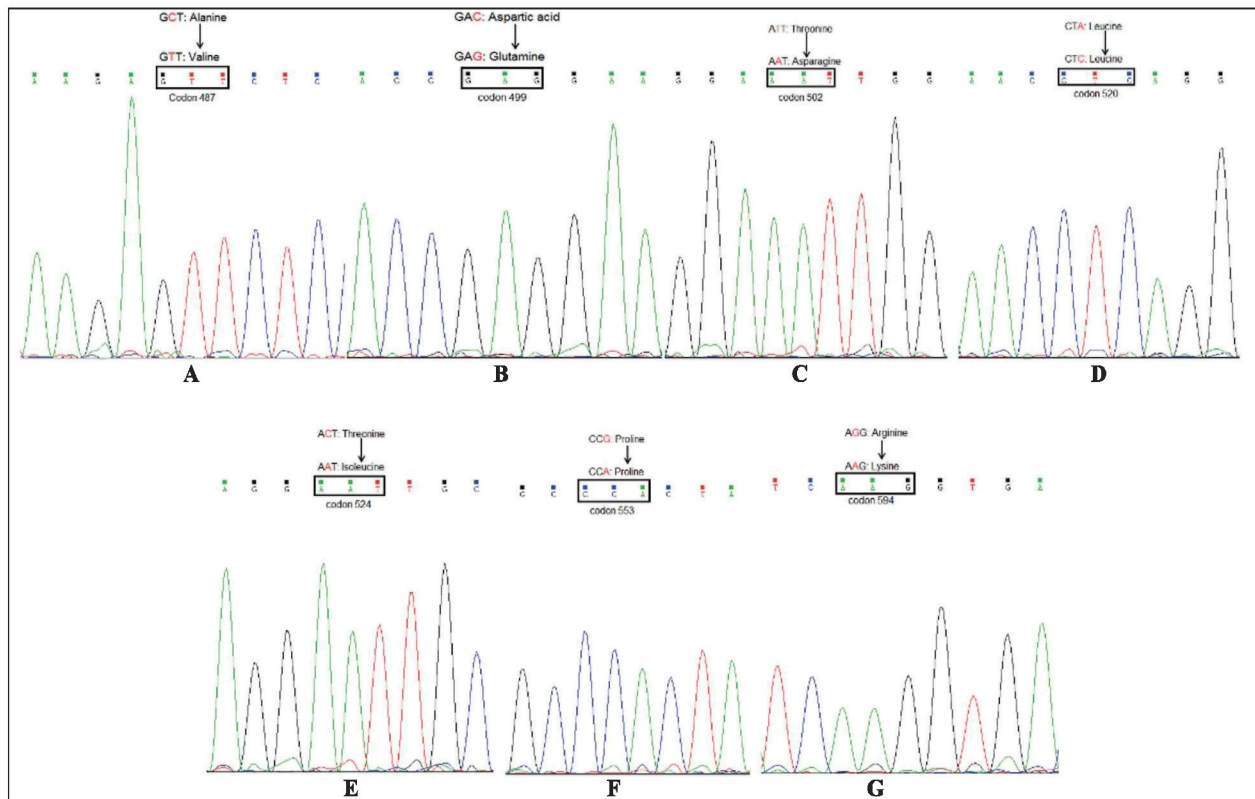


Figure 4. Represented nucleotide substitution consensus changes indicated by T5. Amino acid at position: A) codon 497; B) codon 499; C) codon 502; D) codon 520; E) codon 524; F) codon 553; G) codon 594.

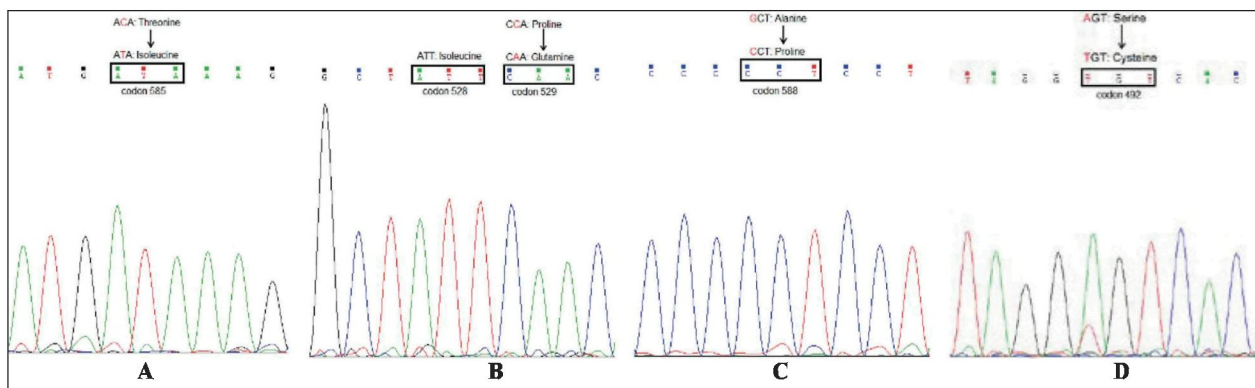


Figure 5. Represented nucleotide/amino acid additional changes: A) codon 585 by T6; B) codons 528 and 529 by T77; C) codon 588 by T77; D) codon 492 by T75.

tions were located on the function domain of EBNA-1 protein, including the dimerization domain, DNA binding domain and transactivation domain, subsequently affecting the function of EBNA-1 related to the viral replication, transcription, or enhancement of oncogenic potential EBV [9,13]. Moreover, the substitution of the amino acid at position 524 (Thr→Ile), led to the subsequent loss of a phosphorylation site in the V-Val subtype [16]. Taken together, the variations of amino acids in V-Val subtypes might be easier maintained at the stage of latent infection.

Given the amino changes at the C-terminal domain of EBNA-1, a total of five patterns were observed. Of these, four of five patterns, named as pattern 2, 3, 4 and 5, were identified as the novel patterns of the V-Val subtype of Vietnamese NPC patients. Notably, a mix of pattern 4 and novel pattern 5 of the V-Val EBNA-1 subtypes, shown in Figure 5(D), were observed. This observation was defined if double signals of base pairs occurred at nucleotide sequence residue 109423. Overall, four novel patterns were identified. However, whether these variations of the novel

Table 2. Nucleotide and amino acid substitution in EBNA-1 C-terminal domain compared to B95-8B sequence.

B95-8	109408	109409	109410	109423	109424	109425	109444	109445	109446	109447	109448	109449	109453	109454	109455	109507	109508	109509	109519	109520	109521
	487			492			499			500			502			520			524		
	G	C	T	A	G	T	G	A	C	G	A	A	A	C	T	C	T	A	A	C	T
	Ala			Ser			Asp			Glu			Thr			Leu			Thr		
T5	.	T	G	A	.	.	.	C	.	T	.	
	Val			*			Glu			*			Asn			+			Ile		
T6	.	T	G	A	.	.	.	C	.	T	.	
	Val			*			Glu			*			Asn			+			Ile		
T77	.	T	G	A	.	.	.	C	.	T	.	
	Val			*			Glu			*			Asn			+			Ile		
T70	.	T	G	A	.	.	.	C	.	T	.	
	Val			*			Glu			*			Asn			+			Ile		
T75	.	T	.	T	.	.	.	G	A	.	.	.	C	.	T	.	
	Val			Cys			Glu			*			Asn			+			Ile		
B95-8	109531	109532	109533	109534	109535	109536	109546	109547	109548	109606	109607	109608	109702	109703	109704	109711	109712	109713	109729	109730	109731
	528			529			533			553			585			588			594		
	A	T	T	C	C	A	C	T	T	C	C	G	A	C	A	G	C	T	A	G	G
	Ile			Pro			Leu			Pro			Thr			Ala			Arg		
T5	G	A	A	A	.	
	Val			*			Ile			+			*			*			Lys		
T6	G	A	A	.	T	A	.	
	Val			*			Ile			+			Ile			*			Lys		
T77	A	A	.	T	.	C	A	.
	*			Gln			*			+			Ile			Pro			Lys		
T70	G	A	A	.	T	.	C	A	.
	Val			*			Ile			+			Ile			Pro			Lys		
T75	G	A	A	.	T	.	C	A	.
	Val			*			Ile			+			Ile			Pro			Lys		

NB: The top four rows correspond to the nucleotide, amino acid position, nucleotide and amino acid of B95-8 prototype sequence. In each row, the upper character denoted the nucleotide that is different to the B95-8 prototype sequence. Conversely, the dot (·) denotes the same nucleotide compared to the reference sequence. The characters below indicate the amino acid written in the three letter code that is different to the reference sequence, while the asterisk (*) indicates the unchanged amino acid, and the plus sign (+) indicates the silent amino acid changes.

Table 3. The comparison between the patterns of the current study and the previous sequences.

Codon	487	492	499	502	520	524	528	529	533	553	585	588	594	
Pattern 1	T5	Val	Ser	Glu	Asn	Leu	Ile	Val	Pro	Ile	Pro	Thr	Ala	Lys
Pattern 2	T6	Ile	.	.	
Pattern 3	T77	Ile	Gln	Leu	.	Ile	Pro	.	
Pattern 4	T70	Ile	Pro	.	.	
Pattern 5	T75	.	/Cys	Ile	Pro	.	
Study 1	GC4	
Study 2		.	.	.	Thr	

NB: The EBNA-1 sequence variants in study 1 [19] and study 2 [16]. The dot (·) denotes the same nucleotide compared to each of the sequences.

V-Val patterns could affect the function of the EBNA-1 protein is uncertain and needs to be further investigated.

Conclusions. In summary, four EBNA-1 subtypes, including V-Val, P-Ala, P-Thr and V-Leu, were identified in 44 of 58 clinical nasopharyngeal biopsy samples, accounting for 75.86%, based on the amino acid at position 487. Of these, the V-Val subtype, accounting for 79.55%, was the preferential subtype in biopsies of NPC. The profile of V-Val subtypes were completely analyzed by sequencing, aligned and compared to the B95-8 prototype sequence (accession #V10555). As a result, five patterns of V-Val subtypes, containing seven consensus changes, including five amino acid changes at positions 487, 499, 502, 524 and 594 and two silent changes at residues 520 and 553, were observed. Notably, four of five patterns, including patterns 2, 3, 4 and 5, were identified as the novel patterns of the V-Val subtype, showed the different changes of amino acid at positions 492, 528, 529, 553, 585 and 588, by comparison with previous studies of V-Val EBNA-1.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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