Original Article

Iran J Ped Hematol Oncol. 2018, Vol 8.No 2, 75-86

Study of *Epstein-Barr virus nuclear antigen* (*EBNA-1*) variations: V-val type preferentially exists in biopsies of nasopharyngeal carcinoma from Vietnamese patients

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Received: 03 December 2017 **Accepted:** 08 February 2018

Abstract

Background: *Epstein-barr virus nuclear antigen 1* (*EBNA1*) plays a crucial role in Nasopharyngeal carcinoma (NPC), the most common cancer of head and neck cancer in Asian countries with high incidents. Sequence variations are of high frequency within the functionally important domains of *EBNA-1*, which have been classified into five subtypes: Phenotype (P)-ala, P-thr, V-val, V-leu and V-pro and are related to geographical location. This study aimed to evaluate the variations of *EBNA-1* in NPC biopsy samples from Vietnamese patients.

Materials and Methods: In this descriptive analytical study, 20 NPC biopsy samples, which were positively confirmed to NPC, were collected from Cho Ray Hospital. Nested PCR – nucleotide sequencing was applied to analyze the carboxy-terminal region of *EBNA-1*. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis, version 5.0, by Neighbor-Joining algorithm.

Results: The variants of *EBNA-1* have been described based on the amino acid signature at codon 487, including V-val (75.0%), P-ala (10%), P-thr (5%), V-leu (5%). Additionally, the phylogenetic results confirmed that the V-val subtype, detected in this current study of the Vietnamese population, was in accordance with previous studies that V-val is almost an exclusive variation in Asian region, especially with Chinese populations and preferentially exists in biopsies of nasopharyngeal carcinoma.

Conclusion: In this initial study, the sub-strains of EBV within V-val subtype of *EBNA-1* was preferential in biopsies of NPC patients. The finding provided the initial data for the potential contribution of *EBNA-1* polymorphisms to etiology of NPC in Vietnamese population.

Keywords: *Esptein-barr virus*, Nasopharyngeal carcinoma, Nuclear antigen, Variations

Introduction

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from epithelial cells that is a striking geographic, ethnic distribution, but is common in Southern China, Southern Asia (1, 2, 3, 4).Worldwide, the incidence and mortality of NPC were 86,691 cases Age-standardized rate)ASR = 1.2/100,000), and 50,831 cases (ASR = 0.7/100,000), respectively (5, 6). Meanwhile, in Vietnam, the incidence and mortality of NPC were 4,931 cases (ASR = 5.4/100,000) and 2,885 cases (ASR = 3.3/100,000), respectively (5, 6). The major etiological factors proposed for NPC pathogenesis are significantly associated with Epstein-Barr virus (EBV) infection, genetic susceptibility and environmental factors (7, 8). EBV, also named human herpes virus 4, a member of Herpesviridae family, has been convincingly shown to be associated to human cancers, such as burkitt's lymphoma, nasopharyngeal carcinoma, etc. (7, 9).Among EBV latent genes, *Epstein–Barr nuclear antigen 1* (*EBNA-1*) gene, which encodes for EBNA-1 protein, has been proved to be the only latent gene, which expressed in viral-associated tumors and crucial for viral latent infection in DNA replication, transcription and partitioning of viral

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genome during cell division by binding to viral latent replication origin regions (10).

Up to now, many studies attempted to identify NPC-specific EBV subtypes based on the sequence variation of *EBNA-1* to display a characteristic geographical prevalence and distribution (11, 12, 13, 14). According to the polymorphism at position 487 of *EBNA-1*, EBV has been classified into two prototypes (P) and three variants (V) subtypes, including P-ala, P-thr, V-val, V-leu and V-pro (15- 18). Many studies indicated that V-val was detected almost exclusively in Chinese populations, and preferentially exists in biopsies of NPC (15, 17, 19). Additionally, unlike other EBV-related diseases, EBV with V-val *EBNA-1* subtype has been proven to be frequently associated NPC, and can be especially detected in oral secretion\s (17). Hence the further analysis of EBV strains, based on *EBNA-1* sequence variation patterns, is considered as an important approach to understand whether there is a disease-related strain specific or geographic distribution of EBV strains and to establish the early reagents for screening, prediction of nasopharyngeal tumorigenesis.

To our knowledge, up to date, the classification of *EBNA-1* subtypes has not been studied in Vietnamese population. Therefore, in current study, NPC biopsy samples were collected from Vietnamese NPC patients, to exam whether certain *EBNA-1* subtypes are preferentially associated with NPC from distinct geographical location.

Materials and Methods

In this historical cohort study, all children

Ethics statement

Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the Cho Ray Hospital, Ho Chi Minh City, Vietnam. The decision number of the permission from Ethical committee: 516/BVCR-HDDD, Cho Ray Hospital, Ho Chi Minh City, Vietnam. All the samples used in this study were agreed by Cho Ray Hospital and obtained from all participants in this clinical trial.

Samples collection, DNA isolation

In this descriptive analytical study, 20 NPC tumor biopsies were collected with informed consent from NPC patients, with the age ranged from30 to 70 years old, mean at 47.7 ± 11.38, at Cho Ray hospital. All samples were submitted to histopathological department and subsequently proved to have NPC by hematoxylin and eosin examination (Fig. 1). Total of genomic DNA was isolated from biopsies by phenol/chloroform method. Biopsies were lysed in lysis buffer (10 mM Tris-HCl pH = 8, 10 mM EDTA, 150 mM NaCl, 2% SDS) containing Proteinase K (0.1 mg/ml). Then, total of genomic DNA was isolated and purified by using standard phenol-chloroform and ethanol precipitation. The quality and purity of DNA extraction were measured by the evaluation of A_{260}/A_{280} proportion. Then, the DNA solution was store at EDTA 0.5M, -20ºC for PCR assay.

Nested-polymerase chain reaction assay, phylogenetic analysis

Nested-polymerase chain reaction (Nested-PCR) and direct sequencing were used to detect the sequence of *EBNA-1* gene. The primers of stage 1 (outer primer) and stage 2 PCR (internal primer) were shown in Table 1. Each stage of PCR was performed in a total of 5 µl DNA template (in case of stage 1 PCR) or 3 µl stage 1 PCR product (in case of stage 2 PCR), 0.75 unit iTaq DNA polymerase (Biorad), 0.5 µM each primer, 7.5 µl MyTaqTM Mix (Bioline). Thermal cycling was initiated at 95ºC for 5 min, followed by 45 cycles of denaturation at 95ºC for 30 secs, annealing at the XºC for 30 secs, extension at 72ºC for 30secs, and a final extension at 72ºC for 10 min (Note: XºC = 55ºC (Stage 1) and 58ºC (Stage 2). Finally, the PCR products of were separated on 2% agarose gel and visualized by ethidium bromide staining.

The sequencing results of *EBNA-1* were checked by Chromas 2.6.4 (Technelysium) to find out whether there were nucleotide

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alterations across amino acid 487 in comparison to the reference sequence of B95-8 prototype (V01555), based on the backbone of the type 1 reference sequence of wild type (NC007605). Alignments between sequences were analyzed using the ClustalW of the Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (20).

For phylogenetic analysis, all isolated sequences were proofread with references to create a homologous database and were subsequently input to MEGA software for multiple alignment and tree reconstruction using NJ algorithm (bootstrap replicate: 1000).

Results

Sequence variations data of *EBNA-1* genes

The Nested-PCR products obtained in distinctly different size, yielded a PCR product of 754 bp, as shown in Fig. 2. The amplification of *EBNA-1* fragments wasdetermined by Sanger sequencing, shown in Fig. 3. Based on the amino acid 487, five *EBNA-1* subtypes were detected. Of these subtypes, V-val was predominant (15 of 20 sequences, counting for 75.0%). P-ala, P-thr, V-leu was presented in the remaining sequences. Notably, bothV-val, P-thr and P-ala were co-detected (1 of 20 sequences, counting for 10%). Additionally, for each isolate, the sequence of *EBNA-1* gene acrossamino acid 487 – 595 (nucleotides 97120 – 97236) was compiled and compared with reference sequence (B95-8 prototype, V01555).

As the results, the sequences with identical consensus mutations were arranged into one group, subsequently, four broad patterns of variations were observed (Table 2).The most common pattern: V-val subtype carried 9 consensus sequence changes, including 7 aa changes at residues 487 (Ala → Val), 499 (Asp → Glu), 502 (Leu → Asn), 524 (Thr → Ile), 528 (Ile → Val), 533 (Leu → Ile), 594 (Arg → Lys) and 2 silent changes at 520 (Leu: CTA → CTC), 553 (Pro: CCG → CCA). This consensus sequence is represented by T69 sequence. The common changes of second pattern: P-ala prototype, which is represented by T72 sequence, were characterized by 5 coding changes at residues 499 (Asp → Glu), 502 (Leu → Asn), 524 (Thr → Ile), 588 (Ala → Pro), 594 (Arg → Lys) and 2 silent changes at 520 (Leu: CTA → CTC), 553 (Pro: CCG → CCA). The third pattern: P-thr prototype, represented by T80, carried 8 consensus changes in residues 487 (Ala → Thr), 492 (Ser → Cys), 524 (Thr → Ile), 563 (Met → Ile), 574 (Val → Gly), 585 (Thr → Pro), 594 (Arg → Lys), 595 (Val → Ala) and 3 silent changes at 499 (Asp: GAC → GAT), 520 (Leu: CTA → CTC), 553 (Pro: CCG → CCA). The forth pattern, represented by T65, was characterized by 9 amino acid changes in residues 487 (Ala → Thr, Val), 492 (Ser → Cys), 502 (Thr →Asn), 524 (Thr → Ile), 574 (Val → Gly), 585 (Thr → Ile), 594 (Arg → Lys) and 3 silent amino changes at 499 (Asp: GAC → GAT), 520 (Leu: CTA → CTC) and 553 (Pro: CCG → CCA).

Phylogenetic analysis of the *EBNA-1* nucleotide sequences

In order to strengthen the classification of above patterns as true strain, all 20 determined *EBNA-1* sequences in current study and 19 sequences in previous studies, including 5 sequences of V-val subtype, 6 sequences of P-thr subtype, 4 sequences of V-leu, 4 sequences of P-ala subtype (table 3), were enrolled into *EBNA-1* dataset and were used to construct the phylogenetic tree. Additionally, the sequence (NC006146) was used as outgroup in phylogenetic construction. According to the resulting phylogenetic tree conducted on MEGA 5.0 using standard parameters, these isolates were segregated and formed five monophyletic group, just the same as the four variant groups and out group, shown in Fig. 4.

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Table I: The primers sequences used in two-stage PCR

Stage	Primer	Sequence (5' – 3')	T_a
1	EBNA-1-1	TAGTCAGTCATCATCATCCG	55°C
	EBNA-1-2	GGGATTTATTCTTTAGTGCG	
2	EBNA-1-3	GCCATTTTTCCACCCTGTAG	60°C
	EBNA-1-4	ATTGAGGGCGTCTCCTAACA	

Table II: Detail EBNA-1 sequence variations in NPC biopsies

***EBNA-1* subtypes**	**No.**	**487**	**492**	**499**	**500**	**502**	**520**	**524**	**528**	**529**
C-terminal		Flanking domain						Core domain		
P-ala	B95.8	GCT	AGT	GAC	GAA	ACT	CTA	ACT	ATT	CCA
		Ala	Ser	Asp	Glu	Thr	Leu	Thr	Ile	Pro
P-ala	T72	…	…	..G	…	.A.	..C	.T.	…	A..
		*	*	Glu	*	Asn	+	Ile	*	Thr
	T96	…	…	..G	…	.A.	..C	.T.	…	…
		*	*	Glu	*	Asn	+	Ile	*	*
P-thr	T80	A..	T..	..T	…	…	..C	.T.	…	…
		Thr	Cys	+	*	*	+	Ile	*	*
V-val	T69	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T70	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T73	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T75	.T.	T..	..G	...	.A.	..C	.T.	G..	...
		Val	Cys	Glu	*	Asn	+	Ile	Val	*
	T77	.T.	...	..G	...	.A.	..C	.T.	...	.A.
		Val	*	Glu	*	Asn	+	Ile	*	Val
	T78	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T79	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*

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	T90	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T91	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T92	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T93	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T94	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T97	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T98	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	Ile	Val	*
	T99	.T.	...	..G	...	.A.	..C	.T.	G..	...
		Val	*	Glu	*	Asn	+	I	Val	*...
V-leu	T76	CT.	T..	..G	..T	.A.	..C	.T.	...	...
		Leu	Cys	Glu	*	Asn	+	Ile	*	*
P-thr/V-val/P-ala	T65	A..	T..	..T	...	.A.	..C	.T.	...	...
		Thr	Cys	+	*	Asn	+	Ile	*	*
		GT.								
		Val								
		G..								
		Ala								

***EBNA-1* subtypes**	No.	533	553	563	574	585	588	594	595	
C-terminal		Core domain								
P-ala	B95.8	CTT	CCG	ATG	GTT	ACA	GCT	AGG	GTG	
		Leu	Pro	Met	Val	Thr	Ala	Arg	Val	
P-ala	T72	…	..A	…	…	…	C..	.A.	…	

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		*	+	*	*	*	Pro	Lys	*
	T96	…	..A	…	…	…	C..	.A.	…
		*	+	*	*	*	Pro	Lys	*
P-thr	T80	…	..A	..T	.G.	C..	…	.A.	.C.
		*	+	Ile	Gly	Pro	*	Lys	Ala
V-val	T69	A..	..A	…	…	.T.	...	.A.	...
		Ile	+	*	*	Ile	*	Lys	*
	T70	A..	..A	…	…	.T.	C..	.A.	...
		Ile	+	*	*	Ile	Pro	Lys	*
	T73	A..	..A	…	…	…	…	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T75	A..	..A	..T	…	.T.	C..	.A.	...
		Ile	+	Ile	*	Ile	Pro	Lys	*
	T77	...	..A	…	…	.T.	C..	.A.	...
		*	+	*	*	Ile	Pro	Lys	*
	T78	A..	..A	…	…	…	…	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T79	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T90	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T91	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T92	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T93	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T94	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*

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	T97	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T98	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
	T99	A..	..A	...	...	...	...	.A.	...
		Ile	+	*	*	*	*	Lys	*
V-leu	T76	...	..A	...	.G.	C..	...	.A.	...
		*	+	*	Gly	Pro	*	Lys	*
V-val/P-thr/P-ala	T65	...	..A	...	.G.	.T.	C..	.A.	...
		*	+	*	Gly	Ile	Pro	Lys	*

Note: Numbers across the top correspond to the amino acid positions under which the B95-8 prototype nucleotide sequence and amino acid is listed. In each row, the upper character denotes the nucleotide which differs to the reference sequence, conversely, the dot (.) denotes the same nucleotide to the reference sequence. The below character indicates the amino acid in three letter code which differs to the reference sequence. On the contrary, the * character indicates the unchanged amino acid, and the + character indicates the unchanged amino acid within the change of the third nucleotide.

Table III: Reference EBNA-1 sequences collected from previous studies

Accession number	Strain	Subtype	Regional distribution	
AY961628	GD1	V-val	China	Asia
HQ020558	GD2	V-val	China	
JQ009376	HKNPC1	V-val	Hong Kong	
KC207813	Akata	V-val	Japan	
KC617875	C666-1	V-val	Hong Kong	
KC207814	Mutu	P-thr	Kenya	Non-Asia
U21202	PA	P-thr	England	
U21198	LA	P-thr	England	
U21205	WW	P-thr	England	
U21200	NL	P-thr	England	
KC440852	K4123-MiEBV	P-thr	USA	
U21196	JF1	V-leu	England	
U21201	P3hr1	V-leu	England	
KC440851	K4123-Mi	V-leu	USA	
DQ279927	AG876	V-leu	Ghana	
V01555	B95-8	P-ala	USA	
AF192740	P-ala	P-ala	Italia	
KT820485	NPC3238	P-ala	Serbia	
NC_007605	Wild type	P-ala	-	-
NC006146	Macacine		Out group	Non-Asia

Note: (-): non-remained.

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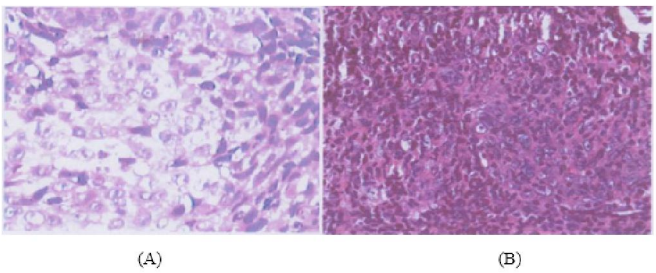

Figure1. Histological examination of (A) undifferentiated carcinoma (WHO type 3); and (B) non-keratinizing squamous cell carcinoma (WHO type 2).

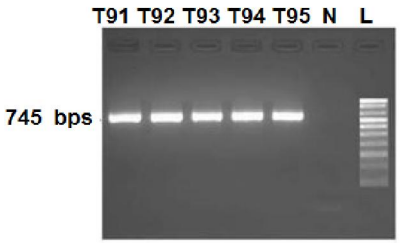

Figure 2. Agarose gel electrophoresis of the PCR products of representative NPC biopsy samples. N: negative control; L: 100-bps DNA marker.

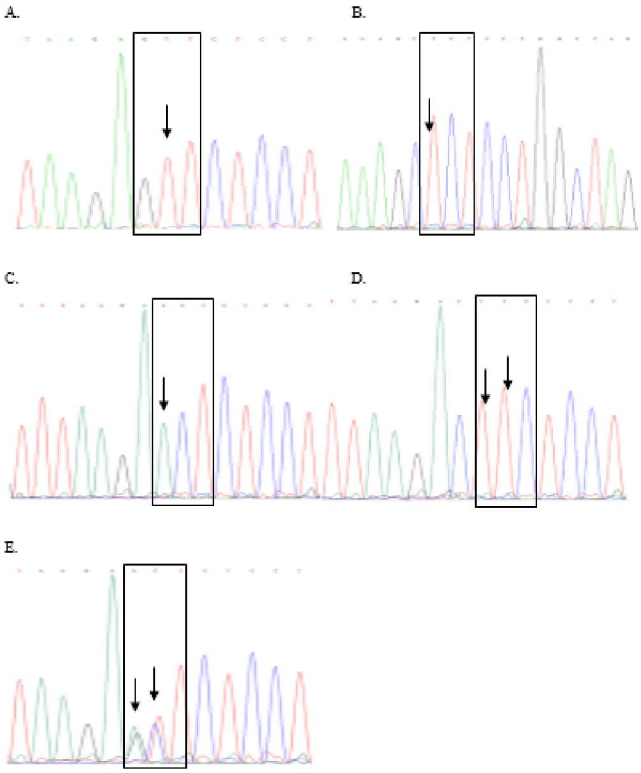

Figure3. Sequence determination of *EBNA-1* subtypes of representative NPC biopsy samples by Sanger sequencing. (A) Sample T70: V-val subtype; (B) Sample T72: P-ala subtype; (C) Sample T80: P-thr subtype; (D) Sample T76: V-leu subtype; (E) Sample T65: P-thr/V-val/P-ala subtype. The amino codon 487 was indicated in square-symbol. The nucleotide variants are indicated in narrow

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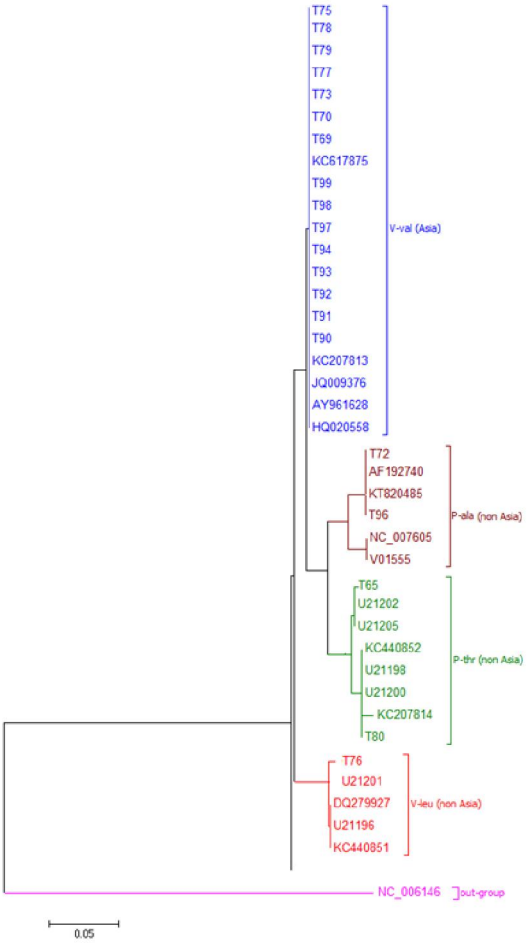

Figure 4. Phylogenetic tree based on sequences of *EBNA-1* gene by Neighbor-Joining method

Discussion

The present study presented the initial study of *EBNA-1* sequence variants evaluation onisolates from the NPC endemic area of Vietnam. EBV has been proven to affect most of global population, counting for more than 90%, only a small percentage of infected individuals develop EBV-associated malignancies, originally form the epithelial of the nasopharynx, EBV-associated NPC. *EBNA-1* is consistently presented in all EBV-associated tumors (21). Besides its role in the replication of EBV genome, *EBNA-1* is an important regulator in the transcription of the viral latent genes (21). In addition, *EBNA-1* has been indicated that to be involved inmany signaling pathways related to cell proliferation and apoptosis, such as STAT1 and TGFβ signaling pathways STAT1 and TGFβ signaling pathways (21, 22). Geographically-associated polymorphism of *EBNA-1* has been observed in previous studies. The classification of *EBNA-1* subtypes was determined at amino acid 487 into P-ala, P-thr, V-val, V-leu and V-pro subtypes, in which V-val was detected almost exclusively in Chinese populations, Hong Kong (15, 16, 17, 18).In current study, the V-val, P-ala, P-thr and V-leu were observed. Of these subtypes, V-val was the common subtype and preferentially detected (counting for 75.0%) in NPC in Vietnamese population. These findings similar to that of previous studies

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demonstrated V-val predominantly exists on Asian population but different from those on Western region (19, 23, 24).Taking together, V-val subtype is the dominant subtype in Asian population.

In present study, we determined the variations in the segment of *EBNA-1* gene across amino acid 487 – 595 (nucleotides 97120 – 97236). Based on the crystal structure analysis, it is divided into two domains, flanking domain (amino acid 461 – 503), which mediated base contacts with DNA binding and core domain (amino acid 504 – 604), which related to the regulation of dimerization, belonged to the C-terminal protein of EBNA-1, based on the background of B95-8 prototype (25, 26). The alteration of amino acid in the flanking domain and core domain of EBNA-1 might affect the ability of DNA binding. In detail, compared to B95-8 prototype, V-val subtype pattern showed 2 aa changes at residues 487, 502 in core domain and 4 aa changes in residues 524, 528, 533, 594 in core domain in C-terminal of EBNA-1. These finding was according to the study of Mai et al (2007) that 4 amino acid mutation fall within the core domain thought to be involved in EBNA-1 dimerization (27). Significantly, the alteration of Ala in B95-8 prototype (P-ala subtype) by Val in the common pattern of V-val subtype (represented by T69 sequence) at codon 487, was also similar to the study Mai et al, 2007, that have been proven to be significant and necessary in initiation of persistent latent infection of EBV in nasopharyngeal cells (27). In Asia, most studies have focused on nasopharyngeal carcinoma, such as Zhang et al (2004) (19) demonstrated that the replacement of threonine by isoleucine at amino acid 524 led to the loss of a phosphorylation in V-val subtype, indicated that V-val subtype infected NPC preferentially led to the susceptibility to a particular EBV isolated in the nasopharynx may exist in NPC development. Thus, taking together, these substitutions of those amino acid in V-val might have changed the ability of *EBNA-1* to anchor cellular chromosome, to act in replication of viral DNA, and even to facilitate immunological evasion, resulting in easier maintenance of latent infection (15, 19), that contributed to nasopharyngeal carcinogenesis.

According to the result of phylogenetic analysis, all isolates were segregated and formed four monophyletic group, including V-val (Asia), P-ala (non-Asia), P-thr (non-Asia), V-leu (non-Asia), which were similar to the classification based on the four broad patterns of variations. The out group was separated into one monophyletic group to indicate as the role of control for the phylogenetic tree analysis. Based on the result, T69, T70, T73, T75, T77, T78, T79, T90, T91, T92, T93, T94, T97, T98, T99 formed the V-val subtype monophyletic group with the sequence from NPC patients in Asian region: China including AY961628 (GD1) and HQ020558 (GD2), Hong Kong including JQ009376 (HKNPC1), KC617875 (C666-1), Japan KC207813 (Akata). These confirmed that V-val subtype, detected in current study - in Vietnamese population, was according to previous studies that V-val almost exclusively in Asian region - Chinese populations and preferentially exists in biopsies of nasopharyngeal carcinoma (15, 17, 19).

Conclusion

In conclusion, this study described the subtypes of *EBNA-1* polymorphisms in Vietnamese patients, including V-val, P-ala, P-thr and V-leu, by the nested-PCR sequencing. Of these EBNA-1 subtypes, V-val was the most prevalent *EBNA-1* subtype (counting for 75%) in Vietnamese NPC patients, which were similar to the previous studies demonstrated that V-val preferentially exists in Asia population, such as Hong Kong, China. Our finding provided the initial data for the potential

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contribution of *EBNA-1* polymorphisms to etiology of endemic NPC in Vietnamese population. In further study, it is necessary to a larger number and various sources, including non-invasive samplesas well as determination of the association V-val subtype with NPC in Vietnamese patients in order to be applied in screening and early prediction of NPC in Vietnamese population.

Acknowledgements

We wish to express our thanks to the research project sponsored by Ho Chi Minh City Open University. We thank all the recruited participants in this work and Dr. Nguyen Huu Dung, Dr. Nguyen Trong Minh and all the staff members of Otorhinolaryngology in Cho Ray hospital, Ho Chi Minh City, for collecting the samples used in these studies.

Conflicts of interest

The authors declared that they have no competing interests.

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