INITIAL STUDY OF SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING OF EPSTEIN-BARR NUCLEAR ANTIGEN 1 (EBNA-1) FROM VIETNAMESE NASOPHARYNGEAL BIOPSY SAMPLES

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ABSTRACT

Background: Epstein-Barr virus nuclear antigen (EBNA1), encoded by *EBNA-1* gene, has been shown as one of the most frequently detected protein in Nasopharyngeal carcinoma (NPC), which the most common and highly incident cancer of head and neck cancer in Asian countries. The geographically-associated polymorphisms of *EBNA-1* have been observed in East-Southern Asia, including Vietnam. The subtype V-val *EBNA-1* has been demonstrated that it may contribute to the oncogenesis of NPC. This current study is initially performed to characterize the variations of EBNA-1 in NPC biopsy samples from Vietnamese patients.

Methods: Nested PCR-sequencing was applied to amplify and characterize the C-terminal region of EBNA-1 gene by the designed oligonucleotide primers.

Results: 10 NPC biopsy samples were enrolled, as the results, only two patterns of EBNA-1 variations: P-ala and V-val were observed. In addition, of these two subtypes, the frequency of V-val and P-ala were determined counting for 80% (8 of 10 cases) and 20% (2 of 10 cases), respectively. It indicated the V-val subtype preferentially exists in biopsy NPC samples, which collected from Vietnamese patients.

Conclusion: We successfully designed the nested PCR-sequencing primer for detection of EBNA-1 variations based on the data collected from previous study. In our initial study, the sub-strain of EBV with V-val subtype of *EBNA-1* infects NPC preferentially to those from biopsies of NPC patients. In further study, it is necessary for a larger number of samples and non-cancerous samples in order to confirm the characteristic of *EBNA-1* variations, as well as determination of the association V-val subtype with NPC in Vietnamese patients.

Keywords: V-val; P-ala; variations; *EBNA-1*; nasopharyngeal carcinoma.

1. Introduction

Epstein-Barr virus (EBV), also called as *human herpesvirus 4*, a member of *Herpesviridae* family, discovered in 1964 by Epstein and Barr, has been convincingly proven to be a significant risk factor for a diverse spectrum of human cancers, including nasopharyngeal carcinoma (NPC). NPC is a highly invasive and malignant tumor that arises in the mucosal epithelium of the nasopharynx, the uppermost region of the pharynx (Sham et al., 1990; Licitra et al., 2003). NPC has a striking geographic and ethnic distribution, especially, exists common in Southern China, Southern Asia (Pathmanathan et al., 1995; McDermott et al., 2001; da Costa et al., 2015). In Vietnam, NPC is one of the most frequent tumor of head and

neck region within the total number were 4,931 cases (ASR = 5.4/100,000) and deaths were 2,885 cases (ASR = 3.3/100,000) (Globocan, 2012). Regarding to EBV, EBV is generally considered to be a single viral species, but, still now, many subtypes and genetic polymorphisms have been identified (Farrell, 2015; Neves et al., 2017). Most recent studies have revealed a diverse spectrum of genetic variations, including many single nucleotide polymorphisms in EBV-related gene which expressed during latent infection. It has been reported that patterns of polymorphisms in the *Epstein-Barr nuclear antigen 1* (*EBNA-1*) that may have correlated to NPC risk (Zhang et al., 2004; Mai et al, 2007; Wang et al., 2010).

EBNA-1 protein, encoded by *EBNA-1* gene, is a DNA-binding nuclear phosphoprotein expressed in all EBV-positive cancer and required for replication and stable maintenance of EBV circular genome (referred to as episome) during latency infected EBV cells (Leight, Sugden, 2000; Frappier, 2015; Frappier, 2015). Up to now, a number of studies have been reported attempts to identify NPC-specific EBV subtypes based on the sequence variation of EBNA-1 to display a characteristic geographical prevalence and distribution. *EBNA-1* sub-strain has been described by comparison with the sequence of prototype B95-8, based on the amino acid alteration at position 487 (Genbank, V01555). 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 (Snudden et al., 1995; Ai et al., 2012; Kwok et al., 2012; Feng et al., 2015). Many studies indicated that V-val was detected almost exclusively in Chinese populations, and preferentially exists in biopsies of nasopharyngeal carcinoma (Gutiérrez et al., 1997; Snudden et al., 1995; Zhang et al., 2004; Ai et al., 2012). Additionally, EBV with an V-val EBNA-1 subtype had been proven to be frequently associated NPC, especially, detected in oral secretion, not in other EBV-related diseases (Gutiérrez et al., 1997).

To our knowledge, up to date, there are still limit research, carried out on the classification of *EBNA-1* subtype in Vietnamese population. We have, therefore, studied in design protocol for genotyping of *Epstein-Barr nuclear antigen 1* (*EBNA-1*) from of NPC biopsy samples collected from Vietnamese NPC patients.

2. Materials and Methods

Ethics statement and NPC biopsy samples collection

Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the 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.

10 NPC tumor biopsies were collected with informed consent from NPC patients at Cho Ray hospital. All of those samples were submitted to histopathological department, subsequently, proved histologically to have NPC. 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 A260/A280 proportion. Then, the DNA solution was stored at EDTA 0.5M, -20^{o}C for PCR assay.

Nested-PCR assay and genotyping

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 15 µl containing 3 µl template DNA (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 95^{o}C for 5 min, followed by 35 cycles of denaturation at 95^{o}C for 30 secs, annealing at the X^{o}C for 30 secs, extension at 72^{o}C for 30 secs, and a final extension at 72^{o}C for 10 min (Note: X^{o}C = 55^{o}C (Stage 1) and 58^{o}C (Stage 2). Finally, the PCR products of were separated on 2% agarose gel and visualized by ethidium bromide staining.

Table 1

The primers sequences used in the current study

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

The sequencing results of *EBNA-1* were checked by Chromas 2.6.4 (Technelysium) to find out whether there were nucleotide alterations across amino acid 487 in comparison to the reference sequence of B95-8 prototype (Genbank accession number V01555), based on the backbone of the type 1 reference sequence of wild type (Genbank accession number NC007605). Alignments between sequences were analyzed using the Clustal W method of the Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0.

3. Results and discussion

In current study, SNP variations of *EBNA-1* in 10 biopsies samples from NPC Vietnamese patients were identified Nested-PCR sequencing assay. The primer of Nested-PCR were cover the Nested-PCR products obtained in distinctly different size, yielded a PCR product of 754 bps-band, shown in Fig. 1A. The amplification of *EBNA-1* fragments was determined by Sanger sequencing, shown in Fig. 1B. In current study, the Nested-PCR was applied and showed the advantages in *EBNA-1* subtyping. Nested PCR, using two sequential sets of primers, could increase the yield, sensitivity and specificity of amplication of the target gene. The first primers bind to the sequence on outside, as expected in standard PCR, which may also bind to the other areas of the DNA sequence. The second primers bind and amplify the products of the first reaction, increasing the sensivity and specicity of amplication.

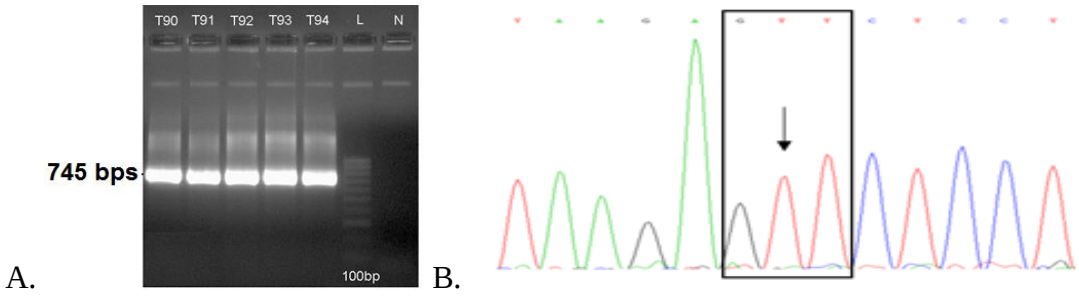

Figure 1. (A) Agarose gel electrophoresis of the Nested-PCR products of representative NPC biopsy samples. N: negative control; L: 100-bps DNA marker; (B) Sequence determination of *EBNA*-1 V-val subtype of representative NPC biopsy samples by Sanger sequencing.

In our study, based on the amino acid 487 of EBNA-1, two EBNA-1 subtypes: V-val and P-ala, were detected. Of these types, V-val was predominant (8 of 10 sequences, counting for 80%). Whereas, P-ala was presented in a few sequences (2 of 10 sequences, counting for 20%). It indicated that V-val subtype preferentially existed in biopsies of nasopharyngeal carcinoma from Vietnamese patients, that was similar to the result of Le et al. (2010). EBNA-1 has been reported to affect several signaling pathways known to be related to cell proliferation and apoptosis, such as STAT1 and TGFβ signaling pathways STAT1 and TGFβ signaling pathways. Moreover, based on the crystal structure analysis, it is divided into two domains, including 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 (Bochkarev et al., 1996; Ceccarell et al., 2000). 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 subtypes 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. In Asia, most studies have focused on nasopharyngeal carcinoma, such as Zhang et al (2004) 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 might 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 (Snudden et al., 1995; Zhang et al., 2004), that contributed to nasopharyngeal carcinogenesis, and significantly presented in NPC.

Additionally, for each isolate, the sequence of *EBNA-1* gene across amino acid 487 – 595 (nucleotides 97120 – 97236) was compiled and compared with reference sequence (B95-8 prototype, accession number: 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). The other 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). These findings similar to that of previous studies demonstrated V-val predominantly exists on Asian population but different from those on Western region (Sandvej et al., 2000; Wang et al., 2002; Zhang et al., 2004). Additionally, in previous study of Le et al. (2010) carried on Vietnamese population, they only showed that the V-val is dominant. However, in current study, the patterns of variations were observed based on the analysis of *EBNA-1* gene across amino acid 487 – 595. These findings provided the initial data for the further study on the molecular screening for subtype of EBNA-1 that is essential in prevention of NPC.

Table 2

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 Ala	AGT Ser	GAC Asp	GAA Glu	ACT Thr	CTA Leu	ACT Thr	ATT Ile	CCA Pro
P-ala	T95	… *	… *	..G Glu	… *	.A. Asn	..C +	.T. Ile	… *	A.. Thr
	T96	… *	… *	..G Glu	… *	.A. Asn	..C +	.T. Ile	… *	… *
V-val	T90	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T91	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T92	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T93	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T94	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T97	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T98	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. Ile	G.. Val	... *
	T99	.T. Val	... *	..G Glu	... *	.A. Asn	..C +	.T. I	G.. Val	... *...

EBNA-1 subtypes	No.	533	553	563	574	585	588	594	595
C-terminal		Core domain							
P-ala	B95.8	CTT Leu	CCG Pro	ATG Met	GTT Val	ACA Thr	GCT Ala	AGG Arg	GTG Val
P-ala	T95	… *	..A +	… *	… *	… *	C.. Pro	.A. Lys	… *
	T96	… *	..A +	… *	… *	… *	C.. Pro	.A. Lys	… *
V-val	T90	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T91	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T92	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T93	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T94	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T97	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T98	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *
	T99	A.. Ile	..A +	... *	... *	... *	... *	.A. Lys	... *

Note: *both nucleotide and amino acid unchanged; +: nucleotide changed, amino acid unchanged.

4. Conclusion

We successfully carried out the Nested-PCR sequencing assay to determine the variations of *EBNA-1* in Vietnamese nasopharyngeal patients. As the results, in our initial study, two EBNA-1 subtypes, V-val and P-thr, counting for 80.0% (8 of 10 sequences), 20.0% (2 of 10 sequences), respectively, were detected. It was indicated that V-val preferentially exists in biopsies of nasopharyngeal carcinoma from Vietnamese patients. In further study, the present findings require extension to a larger number of samples and series on different invasive sources of samples to the profile of EBNA-1 SNPs data in order to establish the early reagents for screening, prediction of nasopharyngeal tumorigenesis■

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