THE INITIAL STUDY OF *EBNA-2* POLYMORPHISMS IN NASOPHARYNGEAL CARCINOMA IN VIETNAM

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ABSTRACT

Epstein-Barr virus (EBV) infection is the main cause of Nasopharyngeal Carcinoma (NPC). *EBNA-2*, one of the most important genes participating in the formation of NPC, also helps EBV evade an attack on the immune system. *EBNA-2* has 4 variants including E2-A, E2-B, E2-C and E-2D, of which E2-A and E2-C are the characterized variants for NPC. This study aimed to evaluate the variations of *EBNA-2* in NPC biopsy samples of Vietnamese patients. This initial study used 10 biopsy samples, which were positively confirmed to NPC, collected from Cho Ray Hospital. Nested PCR – nucleotide sequencing was applied to analyze the variants of *EBNA-2*. The results showed that 8 out of 10 samples, accounting for 80%, were positive to *EBNA-2*. Additionally, only two variants, E-2A and E-2C were detected in our study, in which, E2-A subtype was identified as the predominant subtype. These findings would provide initial data about potential contribution of *EBNA-2* polymorphisms to etiology of NPC in Vietnamese population.

Keywords: E-2A; E-2C; *EBNA-2*; Nasopharyngeal carcinoma; SNPs.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a human malignant disease derived from epithelial cells. There is a striking, geographic and ethnic distribution of this disease which is common in Southern China, Southern Asia (Pathmanathan et al., 1995; McDermott et., 2001; da Costa et al., 2015; Dai et al., 2016). According to Globocan, NPC is considered one of the five most common cancers in men in Vietnam in 2012, of which the number of infected cases is 3,301 (ASR = 7.7/100,000 cases) and of mortality cases is 1,931 (ASR = 4.8/100,000 cases). Besides, there were 1,630 infected cases (ASR = 3.4/100,000 cases) and 954 mortality cases (ASR = 2/100,000 cases) in women. Previous studies showed that major etiological factors proposed for NPC pathogenesis have been significant to the EBV infection (Lo et al., 2004; Tsao et al., 2014; Yang et al., 2005). EBV, also known as human herpes virus 4, a member of Herpesviridae family, has been proven to be significantly associated to many human cancers such as burkitt's lymphoma, gastric carcinoma, nasopharyngeal carcinoma, and so on (Lo et al., 2004; Tsao et al., 2014). Understanding the role of EBV latent genes is essential for identifying the mechanism underlying EBV-induced cell transformation and immune evasion (Ko, 2015). Among them, *EBNA-2* has been shown to be essential for the infection of EBV and existed in the cellular. Numerous studies have long been trying to identify NPC-specific EBV subtypes based on the sequence variation of *EBNA-2* to display a characteristic geographical prevalence and distribution. The *EBNA-2* polymorphism was classified into four subtypes including E2-A, E2-B, E2-C and E2-D. To the best of our knowledge, there has been no research on the classification of

EBNA-2 subtype in Vietnamese population. This study, therefore, aims to analyze NPC biopsy samples collected from Vietnamese NPC patients for the first time to examine whether there is a remarkable association between certain *EBNA-2* subtypes and NPC from distinct geographical location.

2. Materials and method

2.1. Ethics statement

Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the Cho Ray Hospital, Ho Chi Minh City, Vietnam. The permit from Ethical committee was under decision number 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.

2.2. Sample collection

10 NPC tumor biopsies were collected with informed consent from NPC patients at Cho Ray hospital. All samples were submitted to histopathological department and then, proved histologically to have NPC by hematoxylin and eosin examination. Total genomic DNA extraction was isolated from biopsy samples by using Phenol/chloroform method. The samples 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 genomic DNA extraction 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, -20^{o}C for PCR assay.

2.3. Nested – Polymerase Chain Reaction assay (Nested – PCR)

Nested – PCR and direct sequencing were used to detect the sequence of *EBNA-2*. The primers of stage 1 (outer primer) and stage 2 (internal primer) were shown in Table 1. Total volume reaction is 15 µl containing 2 µl DNA isolation (for stage 1) or 2 µl PCR product of stage 1 (for stage 2), 0.75 unit iTaq DNA polymerase, 0.5 µl each primer, 7.5 µl MyTaqTM Mix and water up to 15 µl. Thermal cycling was set up at 95^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at the 55^{o}C for 30 seconds, extension at 72^{o}C for 30 seconds, and a final extension at 72^{o}C for 10 min. Finally, The PCR products were expressed by electrophoresis on 2% agarose gel and DNA was dyed with ethidium bromide.

Table 1

The primer sequences used in this study

Region	Stage	Primer	Sequence (3’ – 5’)	Ref
EBNA2-A (163-294)	1	E2A-OF	GCTATGCGAATGCTTTGG	*(Wang et al., 2012)*
		E2A-OR	GAGTCTTAGAGGGTTGCG	
	2	E2A-IF	CTATGCGAATGCTTTGGA	
		E2A-IR	TTGTTGGTCGTTGATGAC	
EBNA2-C (357-486)	1	E2C-OF	AGAACCACGGTCCCCGACTGTA	
		E2C-OR	TGCTGAGAGCAAGGCACCAATT	
	2	E2C-IF	ACGGTCCCCGACTGTATTTTAT	
		E2C-IR	TTTTGGCAAGCCTTCCTT	

3. Results and Discussion

3.1. Sequence variation data of EBNA-2 in NPC biopsy samples

In this study, nested-PCR was used to amplify the target sequence. As a result, 8 out of 10 samples, accounting for 80%, were positive to *EBNA-2*, which yielded a PCR product of 551 bps-band and 788 bps-band as shown in Figure 1. The amplification of *EBNA-2* fragments, including *EBNA-2A* and *EBNA-2C*, was determined by Sanger sequencing as shown in Figure 2.

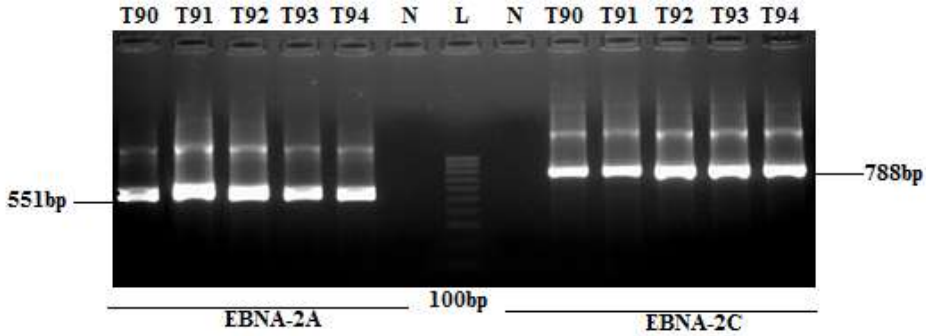

Figure 1. Agarose gel electrophoresis of the PCR products of representative NPC biopsy samples. N: negative control. Ladder: 100 bps

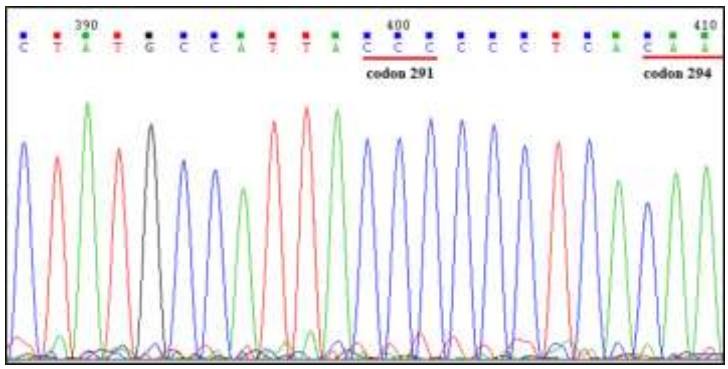

Figure 2. Sequence determination of *EBNA-2A* of representative NPC biopsy samples by Sanger sequencing

Table 2

Variant positions of *EBNA-2*

Sub types	Amino Acid	151	160	163	165	185	195	200→217	201
		Self-association domain							
	WT	ATT Ile	CCT Pro	AGG Arg	GTC Val	CAA Gln	ATG Met	51 nucleotide 18 a.a	ACC Thr
E2-A	T79	--G Met	--C +	GT- Val	--A +	-G- Arg	-C- Thr	--- *	--T +
	T80	--G Met	--C +	GT- Val	--A +	-G- Arg	--- *	--- Del	--- *
	T86	--G Met	--C +	GT- Val	--A +	-G- Arg	-C- Thr	--- *	--T +

Sub types	Amino Acid	151	160	163	165	185	195	200→217	201
		Self-association domain							
	WT	ATT Ile	CCT Pro	AGG Arg	GTC Val	CAA Gln	ATG Met	51 nucleotide 18 a.a	ACC Thr
	T90	--G Met	--- *	GT- Val	--A +	-G- Arg	--- *	--- Del	--- *
	T91	--G Met	--C +	GT- Val	--A +	-G- Arg	-C- Thr	--- *	--T +
	T97	--G Met	--- *	GT- Val	--A +	-G- Arg	-C- Thr	--- Del	--- *
E2-C	T93	--- *	--- *	-T- Met	--A +	--- *	--- *	--- *	--- *
	T94	--- *	--- *	-T- Met	--A +	--- *	--- *	--- *	--- *

Sub types	Amino Acid	237	241	245	246	279	280	357	358
		NLS							
	WT	GAA Glu	-	CCA Pro	CGC Arg	CCG Pro	ACT Thr	CAA Lys	GGG Gly
E2A	T79	--- *	CTC Leu	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
	T80	-C- Ala	--- *	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
	T86	--- *	CTC Leu	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
	T90	-C- Ala	--- *	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
	T91	--- *	CTC Leu	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
	T97	-C- Ala	--- *	-A- Gln	--T Ser	--A +	-A- Asn	--- *	--- *
E2-C	T93	--- *	--- *	--- *	--- *	--- *	--- *	--- Del	--- Del
	T94	--- *	--- *	--- *	--- *	--- *	--- *	--- Del	--- Del

Sub types	Amino Acid	370	383	417	470	476	486	490	496	509
		NLS	TAD domain					-		
	WT	**CCT Pro**	**CCT Pro**	**ACG Thr**	**TCA Ser**	**GAG Glu**	**ATC Ile**	**CCC Pro**	**ACC Thr**	**AAC Asn**
E2-A	T79	--A +	--- *	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
	T80	--A +	--- *	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
	T86	--A +	--- *	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
	T90	--A +	--A +	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
	T91	--A +	--- *	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
	T97	--A +	--A +	--A +	--T +	-G- Gly	-C- Thr	A-- Thr	--- *	G-- Asp
E2-C	T93	--A +	--- *	--- *	--- *	--- *	--- *	--- *	-A- Asn	--- *
	T94	--A +	--- *	--- *	--- *	--- *	--- *	--- *	-A- Asn	--- *

Note: (-) a position with the same nucleotide as Wild type sequence; (*) a position with the same amino acid as Wild type sequence; (+) a position with the same amino acid as Wild type on silent mutations, and (Del) a position with deleted amino acid.

EBNA-2 is one of the first latent proteins detected after an EBV infection and primarily upregulates the expression of viral and cellular genes. As the functions of *EBNA-2*, it interacts with other transcription factors associated in the Notch signaling pathway (Henkel et al., 1995; Hsieh et al., 1996). The variants of *EBNA-2* has evidently involved in the NPC carcinoma tumorigenesis. According to previous studies on *EBNA-2* gene polymorphisms in gastric carcinoma and NPCs, the variant of E-2A was significantly detected only in NPC and more important in the pathogenesis of NPC (Wang et al., 2012). As for the *EBNA-2* protein structure, the three important domains for transcription regulation function of *EBNA-2* including self-association domain (122–232 aa), trans-activation domain (TAD; 431–487 aa) and nuclear localization signals (NLS; 244–378 aa, and 466–483 aa) (Cohen et al., 1991) were sequenced and analyzed in this study. In detail, experimental samples of E2-A variants were divided into two distinct groups with group 1 having the characteristic of the mutation loss of 51 bp at 49103 and group 2 containing the three-nucleotide CTC insertion mutation at 49136. The DNA pattern of E-2A group 1 subtype carried 11 amino acid changes at residue 151 (ATT:Ile → ATG:Met), 163 (AGG:Arg → GTG:Val), 186 (CAA:Gln → CGA:Arg), 237 (GAA:Glu → GCA:Ala), 245 (CCA:Pro → CAA:Gln), 246 (CGC:Arg →

CGT:Ser), 280 (ACT: Thr → AAT: Asn), 476 (GAG:Glu → GGG:Gly), 486 (ATC:Ile → ACC:Thr), 490 (CCC:Pro → ACC:Thr), 509 (AAC:Asn → GAC:Asp) and one deletion at 200-217 (Del), represented by T80 sequence. The second pastern of group 2, which was represented by T69, carried 11 amino acid changes at residue 151 (ATT:Ile → ATG:Met), 163 (ACG:Arg → GTG:Val), 185 (CAA:Gln → CGA:Arg), 195 (ATG:Met → ACG:Thr), 245 (CCA:Pro → CAA:Gln), 246 (CGC:Arg → CGT:Ser), 280 (ACT:Thr → AAT: Asn), 476 (GAG:Glu → GGG:Gly), 468 (ATC:Ile → ACC:Thr), 490 (CCC:Pro → ACC:Thr), 509 (AAC:Asn → GAC:Asp) and one insertion in 241 (CTC: Leu). In this study, the variants E2-A was identified as the predominant subtype in our samples, which was also characterized as the frequent subtype in other Asian regions such as Hong Kong (KwoK et al., 2014) and southern China (Wu et al., 2015).

4. Conclusion

In conclusion, this study described the subtypes of *EBNA-2* polymorphisms in Vietnamese NPC biopsy samples, including E-2A and E-2C. Of the *EBNA-2* subtypes, E-2A was the most prevalent *EBNA-2* subtype in Vietnamese patients, in which two different patterns were identified. Considering the shortcomings of this work, our finding provided the initial data for the potential contribution of *EBNA-2* polymorphisms to etiology of endemic NPC in Vietnamese population. Larger number and direct comparison of various sample sources such as throat washing should be included in future research. It will be very useful to indicate the sample type specificity of genotyping changes to apply in genetic screening of NPC in Vietnamese population■

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References

Da Costa, V. G., Marques-Silva, A. C. & Moreli, M. L. (2015). The Epstein-Barr virus latent membrane protein-1 (LMP1) 30-bp deletion and XhoI-polymorphism in nasopharyngeal carcinoma: a meta-analysis of observational studies. *Syst Rev*. 13, 46.

Dai, W., Zheng, H., Cheung, A. K. & Lung, M. L. (2016). Genetic and epigenetic landscape of nasopharyngeal carcinoma. *Chin Clin Oncol*, *5(2)*, 16-28.

Henkel, T., Ling, P.D., Hayward, S.D., Peterson, M.G. (1994). Mediation of Epstein-Barr virus *EBNA2* transactivation by recombination signal binding protein J kappa. *Science*, *265*, 92–95.

Hsieh, J.J., Henkel, T., Salmon, P., Robey, E., Peterson, M.G., Hayward, S.D. (1996). Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus *EBNA2*. *Mol Cell Biol*, *16*, 952–959.

Ko YH (2015). EBV and human cancer. *Exp Mol Med 47*: e130.

Kwok, H., Wu, C.W., Palser, A.L., Kellam, P., Sham, P.C., Kwong, D.L., Chiang, A.K. (2014). Genomic diversity of Epstein-Barr virus genomes isolated from primary nasopharyngeal carcinoma biopsy samples. *J Virol*, *88*, 10662–10672.

Lo, K.W., To, K.F., Huang, D.P. (2004). Focus on nasopharyngeal carcinoma. *Cancer Cell, 5*, 423–428.

McDermott, A. L., Dutt, S. N. & Watkinson, J. C. (2001). The aetiology of nasopharyngeal carcinoma. *Clin Otolaryngol Allied Sci, 26(2)*, 82-92.

Pathmanathan, R., Prasad, U., Sadler, R., Flynn, K. & Raab-Traub, N. (1995). Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. *N Engl J Med, 333*, 693-698.

Tsao, S.W., Yip, Y.L., Tsang, C.M., Pang, P.S., Lau, V.M., Zhang, G., Lo, K.W. (2014). Etiological factors of nasopharyngeal carcinoma. *Oral Oncol, 50*, 330–338.

Wang, X., Wang, Y., Wu, G., Chao, Y., Sun, Z., and Luo, B. (2012). Sequence analysis of Epstein-Barr virus EBNA-2 gene coding amino acid 148-487 in nasopharyngeal and gastric carcinomas. *Virol. J, 9*, 49.

Wu, G., Liu, X., Liu, S., Shu, J., Sun, Z. and Luo, B. (2015). Epstein-Barr Virus EBNA-2 Polymorphic Patterns in Nasopharyngeal Carcinoma in Southern China. *Intervirology, 58*, 386–392.

Yang, X.R., Diehl, S., Pfeiffer, R., Chen, C.-J., Hsu, W.-L., Dosemeci, M., Cheng, Y.-J., Sun, B., Goldstein, A.M., Hildesheim, A., et al. (2005). Evaluation of risk factors for nasopharyngeal carcinoma in high-risk nasopharyngeal carcinoma families in Taiwan. Cancer Epidemiol. Biomark. Prev. Publ. Am. Assoc. *Cancer Res. Cosponsored Am. Soc. Prev. Oncol, 14*, 900 – 905.