RESEARCH ARTICLE

Molecular Screening for Epstein-Barr virus (EBV): Detection of Genomic *EBNA-1*, *EBNA-2*, *LMP-1*, *LMP-2* Among Vietnamese Patients with Nasopharyngeal Brush Samples

Thuan Duc Lao[1,2], Dung Huu Nguyen[3], Trong Minh Nguyen[3], Thuy Ai Huyen Le[2]*

Abstract

Epstein-Bar virus (EBV) is considered to be intimately associated with development of nasopharyngeal carcinoma (NPC), the most common and high incidence cancer of the head and neck in Asian countries, especially in Vietnam. In this study, we validated associations between highly conserved segments of the EBV genome, including *EBNA-1*, *EBNA-2*, *LMP-1*, *LMP-2* and nasopharyngeal cancer, with the aim of applications with non-invasive brush samples for early diagnosis and as a prognostic biomarker. The polymerase chain reaction (PCR) was performed to assess the presence of *EBNA-1, EBNA-2, LMP-1, LMP-2* in nasopharyngeal brush samples from Vietnamese NPC patients and non-cancer controls. Positive rates for *EBNA-1*, *EBNA-2*, *LMP-1*, *LMP-2* were 46.3%, 49.5% and 45.3%, and 47.4%, respectively, in cancer cases, much higher than the low frequencies found in non-cancer samples. Notably, at least one of the four (PI $\geq$ 0.25) was found in 57. 9%. Significance was reached when computing the odds ratio (OR) and relative risk (RR). Our detection of candidate genes. in nasopharyngeal brush samples collected from Vietnamese patients reduces the need for invasive biopsies and fulfills the characteristics of a non-invasive, specific screening method suitable for routine, NPC risk assessment for EBV-infected populations. Notably, this procedure may be useful for confirmatory screening with large oral brush swabs.

Keywords: *EBNA-1- EBNA-2- LMP-1- LMP-2-* nasopharyngeal swab

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Introduction

Nasopharyngeal carcinoma (NPC) has a striking and geographic and ethnic distribution, which encountered in Asian region, gravitating toward Southeast Asia, especially in China and Vietnam (Pathmanathan et al., 1995; da Costa et al., 2015). According to data provide by GLOBOCAN 2012, in the world, the total of number of NPC was 86,691 cases (Age-standardized rate – ASR = 1.2/100,000), while the number of death was 50,831 (ASR = 0.7/100,000). Among these cases, Vietnam was one of countries contributed to the most to these indices, because the total number was 4,931 cases (ASR = 5.4/100,000) and deaths was 2,885 cases (ASR = 3.3/100,000) (3).

Notably, the symptoms of NPC were unclear, such as hearing loss, nosebleeds, headache, trouble opening the mouth, etc., for this reason, that NPC is commonly diagnosed at a late stage (stage 3 or 4) (Epstein, Jonea, 1993; Hao et al., 2014). Despite growing evidences, NPC is still characterized by late diagnosis in regional sites such as neck lymph nodes or in distant sites as metastases (Skinner, Van Hasselt, 1990; Mao et al., 2009). Therefore, the major obstacle to early diagnosis and screening of NPC is the different access due to the deeply seated location of nasopharynx, as well as the unclear presenting symptoms. Thus, it is necessary to have an early diagnosis and screening to achieve favorable treatment and increasing of patient's survival.

According to the etiology, NPC is considered to be significantly associated with Epstein-Barr virus (EBV) infection, which is a gamma herpes virus discovered in 1964 by Epstein and Barr, taxonomically called Human herpes virus 4 (Epstein et al., 1964; Lo et al., 2004; Frappier et al., 2012; Lung et al., 2014). EBV infection has been considered to be an early event, pre-requisite step in nasopharyngeal tumorigenesis, played an important role in the carcinogenesis of the disease, and are presented in the majority of nasopharyngeal carcinoma (Pathmanathan et al., 1995; Yap et al., 2017; Yip et al., 2010; Chan, Wong, 2014). Since then, viral infection has been received much attention and represented a prospective biomarker for diagnosis and screening of NPC.

For the past decades, owing to achieve early cancer detection, numerous molecular studies were carried out on NPC, which mainly focused on polymerase chain reaction (PCR), in situ hybridization, real-time PCR methods to detect the presence of EBV in NPC. Circulating cell-free Epstein-Barr virus DNA in plasma or in the peripheral

*[1]Faculty of Biology and Biotechnology, University of Science, Vietnam National University, [2]Faculty of Biotechnology, Ho Chi Minh City Open University, Ho Chi Minh City, [3]Cho Ray Hospital, Vietnam. *For Correspondence: thuy.lha@ou.edu.vn*

blood has been considered as potential tool for screening of NPC (Lo et al., 1999; Han et al., 2002). However, instability of plasma DNA has been demonstrated having a low sensitivity, given examples, according to Breda et al., (1999), in the cases of samples from the peripheral blood of NPC patients, 53.1% of positive EBV cases were found, and presents restrictive specimen preservation parameters that creates practical and logistical challenges (Lo et al., 1999; Ng et al., 2014). To generate the accurate and potential diagnosis and screening test based on detection the presence of EBV DNA, the trans-oral brushing of the nasopharynx for the acquisition of NP epithelial samples has been focused, according to Ng et al., (2014), they found that a sensitivity of 98.9%, specificity of 99.3%, positive predictive value (PPV) of 96.9%, and negative predictive value (NPP) of 99.7% for NP Screen in detecting NPC by Q-PCR EBV *EBNA-1* (Epstein–Barr nuclear antigen 1) determinations. Thus, the trans-oral brushing system fulfills the characteristics of a non-invasive, sensitive, specific detection method suitable for routine, large-scale ambulatory NPC risk assessment for high-risk NPC populations (Han et al., 2002; Ng et al., 2014). Considerably, while the molecular detection of EBV genome in various biological non-invasive samples has been supported by wide range of developed countries, concerning to Vietnam, developing country within the high accidence of NPC in population, the study of EBV genome was still limited. Therefore, the purpose of current study was to analyze the presence of EBV in oral bushing nasopharyngeal samples of NPC patients for generation of non-invasive method detection and screening for NPC, which will be facilitating applied in Vietnamese population.

Materials and Methods

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. The patients are required to be agreed and sign on the consent forms.

Sample collection

A total of 95 nasopharyngeal brushing samples were archived and admitted from the Cho Ray Hospital, Vietnam, and collected from June, 2015 to December, 2015. All of those samples were submitted to histopathological department, subsequently, ninety-five were proved histologically to have NPC and 95 samples were negative for carcinoma. All the samples were placed in 1.5-ml tubes containing PBS buffer and stored at -20oC for further experiments.

DNA extraction

Total of genomic DNA was isolated from NBS by phenol/chloroform method. Cells obtained from NBS 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 (BIO-RAD SmartSpec Plus UV/Visible Spectrophotometer). Then, the DNA solution was store at EDTA 0.5mM, -20oC for PCR assay.

Polymerase chain reaction assay

The primers, which were used in current study, were obtained by previous study: *EBNA-1* (forward primer: 5'-GTCATCATCATCCGGGTCTC-3' and reverse primer: 5'-TTCGGGTTGGAACCTCCTTG-3'); EBNA-2 (forward primer: 5'-CAGGTACATGCCAACAACCTT-3' and reverse primer: 5'- CCAACAAAGATTGTTAGTGGAAT-3') (Yap et al., 2007); LMP-1 (forward primer: 5'-CAGTCAGGCAAGCCTATG-3' and reverse primer: 5'-CTGCTTCCGGTGGAGATG-3'); LMP-2 (forward primer: 5'- AGCTGTAACTGTGGTTTCCATGAC-3' and reverse primer: 5'- GCCCCCTGGCGAAGAG -3') (Ryan et al., 2004) gene for identification of EBV genomic DNA. Additionally, the amplification of human beta-actin gene (forward primer: 5'-ATCATGTTTGAGACCTTCAACAC-3' and reverse primer: 5'- CATCTCTTGCTCGAAGTCCAG-3') acted as an internal marker for the presence of intact genomic DNA (Yap et al., 2007).

The amplification was done in a total volume of 15 µl, containing 1 µg DNA template. Thermal cycling was initiated at 95oC for 5 min, followed by 40 cycles of denaturation at 95oC for 30 secs, annealing at the XoC for 30 secs, extension at 72oC for 30sec, and a final extension at 72oC for 10 min (Note: X = 63.0oC, 61.5oC, 65.5oC, 65.0oC for *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2*, respectively). The PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide, additionally, confirmed by DNA sequencing.

Statistical analysis

Statistical analyses were performed by Medcalc® Version 12.7.0.0. The frequencies of *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* were calculated. The association between the presence. Chi-squared test was used to compare the frequencies of categorical variables between groups. The differences of frequencies of *EBNA-1*, EBNA-2, LMP-1 and LMP-2 among groups were considered statistically significant for $p \leq 0.05$. Moreover, the association between the presence of the presence of both four candidate genes and risk of NPC was estimated by computing odds ratios (OR), relative risk (RR) and 95% confidence intervals (CI).

Results

Status of the presence of EBNA-1, EBNA-2, LMP-1 and LMP-2

The frequencies of *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* in clinical samples were examined by PCR method.

Table 1. Validity Data for Individual Gene Detection in Nasopharyngeal Cancer Specimens and Non-Cancerous Samples

		EBNA-1	EBNA-2	LMP-1	LMP-2	Four genes combination*
Nasopharyngeal specimens	P (n(%))	44 (46.32)	47 (49.47)	43 (45.26)	45 (47.37)	55 (57.89)
	N (n(%))	51 (53.68)	48 (50.53)	52 (54.74)	50 (52.63)	40 (42.11)
Non-cancerous specimens	P (n(%))	1 (1.05)	0 (0.00)	0 (0.00)	1 (4.17)	2 (2.11)
	N (n(%))	94 (98.95)	95 (100.00)	95 (100.00)	23 (95.83)	93 (97.89)
p value		*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*

*Note, Four gene combination with PI (PCR value) ≥ 0.25.

Overall, the frequencies of *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* in NPC samples were 46.32% (44 of 95 samples), 49.47% (47 of 95 samples), 45.26% (43 of 97 samples), and 47.37% (45 of 95 samples), respectively. In non-NPC brushing samples, the frequencies were 1.05% (1 of 95 samples), 0% (0 of 95 samples), 0% (0 of 95 samples) and 1.05% (1 of 95 samples) for *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2*, respectively. The presence of EBV DNA in nasopharyngeal samples was found to be significant association to NPC ($p < 0.05$) (Table 1).

The PCR product sizes were different which is easy to be distinguished on agarose gel, shown in Figure. 1. The *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* forward and reverse primers yielded a PCR product of 269, 219, 106 and 69 bps, respectively. The human beta-actin PCR product of 319 bps gave indication on the quality of the extracted DNA. The signals of peaks in PCR product sequencing were good for nucleotide reading (Data not shown). According to Blast results, candidate genes' sequence was similar to Human Herpesvirus 4 (Epstein-Barr virus). Individually, *EBNA-1* sequence was similar to KP735248, within Total score = 462, Ident = 100% and E-value = 1e-126. EBNA-2 sequence was similar to accession number LN27567, within total score = 361, Ident = 100% and E-value = 3E-96. LMP-1 and LMP-2 were similar to sequence with accession number KT001103, within Total score = 150, Ident = 100% and E-value = 2e-33 (in the case of LMP-1) and total score = 91.7, Ident = 100% and E-value = 7e-16 (in the case of LMP-2).

In this study, the PCR index value (PI) was calculated, shown in Table 2. In the case of PI ≥ 0.25, which meant that at least one of four genes was detected, also indicated that the frequency of all four candidate genes combination in cancerous samples showed the increasing of frequency within at least one gene of them was detected, counting for 57.89% (55 of 95 samples). Moreover, in the non-cancerous samples, the frequency of all four candidate genes combination was 2.11% (2 of 95 samples). Additionally, the significant correlation between detection of four genes combination and nasopharyngeal cancer was observed ($p < 0.0001$).

Table 2. Calculation of PI Value in Nasopharyngeal Samples

PI (PCR index)	n (%)
0 (none of detection)	40 (42.1)
0.25 (one gene detected)	7 (7.37)
0.5 (two genes detected)	2 (2.11)
0.75 (three genes detected)	16 (16.84)
1 (four genes detected)	30 (31.57)
≥ 0.25 (at least one gene detected)	55 (57.89)

Odds ratio, relative risk for candidate genes detection

In this study, the odds ratio and relative risk value were

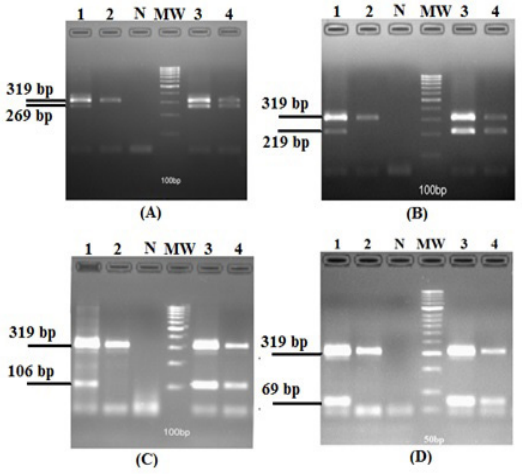

Figure 1. Agarose Gel Electrophoresis Showing the Present of (A) EBNA-1; (B) EBNA-2; (C) LMP-1, (D) LMP-2; and beta-actin in representative samples. N, Negative control; MW, ladder; (1) (2) (3) (4), clinical samples.

Table 3. Calculation of OR and RR Value

	EBNA-1	EBNA-2	LMP-1	LMP2	Four genes
OR	81.1	187.06	158.26	84.6	63.94
p	p < 0.0001	p = 0.0003	p = 0.0004	p < 0.0001	p < 0.0001
95%CI	10.85 – 606.01	11.29 – 3100.13	9.55 – 2623.41	11.32-632.11	14.87 – 274.97
RR	44	95	87	45	27.5
p	p = 0.0002	p = 0.0013	p = 0.0016	p = 0.0001	p < 0.0001
95%CI	6.19 – 312.89	5.94 – 1519.12	5.43 – 1393.06	6.33 – 319.84	6.96 – 109.52

computed between EBV genomic DNA detection and nasopharyngeal cancer, shown in Table 3. Additionally, in the case of candidate genes combined, Sp (Specificity), Se (Sensitivity), Pp (positive predictive value), Np (Negative predictive value) were 57.89%, 97.89%, 96.49% and 69.92%, respectively.

Discussion

Due to unclear symptoms, nasopharyngeal cancer often presents in the last stage when first diagnosis. The detection of NPC at the early stage certainly led to improved treatment and outcome (Epstein, Jones, 1993; Hao et al., 2004). Therefore, there is a challenge for finding a simple and non-invasive methods access to NPC. Finding the simple, non-invasive access to the NPC has been described in previous studies by obtaining various, including plasma, peripheral blood, nasopharyngeal brushing samples, etc. for EBV DNA analysis. EBV consists a double-stranded 184-kbp-long DNA enclosed in a protein capsid and that encode more than 85 genes. The EBV genomic organization that code for EBNAs (Epstein-Barr virus nuclear antigens) including *EBNA-1*, 2, 3A, 3B, 3C, and LP; *LMP-1*, 2A and 2B (latent membrane protein); two small EBERs (non-coding nuclear RNAs) (Young et al., 1988; Hammerschmidt, Sugden, 2013). Individually, *EBNA-1* plays an important role of EBV infection. As function, *EBNA-1* is essential for EBV immortalization of cell and responsible for EBV DNA episome replication, segregation and persistence of viral genome. *EBNA-1* coded-protein is only protein which expressed in both latent and lytic modes of infection (Sivachandran et al., 2012; Hammerschmidt, Sugden, 2013). Additionally, *EBNA-1* is necessary to maintain EBV genome in circular episome with multicopies in the infected cell (Thompson, Kurzrock, 2004). Besides, EBNA-2 is co-expressed soon after EBV infection, and is essential for cell transformation leading to carcinogenesis. Primarily, it is directly associated to the switching of transcription from Wp promoter which is the first promoter to be activated immediately after EBV infection (Thompson, Kurzrock, 2004; Sivachandran et al., 2012; Hammerschmidt, Sugden, 2013). Concerning to LMPs, LMP-1 (Latent membrane protein-1) encoded its latent membrane protein within function as a viral mimic of the TNFR family member, CD40, engaging a number of signaling pathways, such as NF-κB, JNK, p38 pathway, etc., that induce morphological and phenotypic alterations in epithelial cells (Hao et al., 2004; Dawson et al., 2012). The function of LMP-2 is considered as playing important role in carcinogenesis by driving EBV into latency (Thompson, Kurzrock, 2004; Dawson et al., 2012). Previous attempts of NPC diagnosis by various molecular methods, which were based on in situ hybridization (ISH), Southern blotting, or RISH for detecting EBERs, etc. In this study, the samples obtained by brushing directly at nasopharynx are predominately intact the epithelial cells samples from the nasopharynx surface. Therefore, the presence of DNA in exfoliating cells has offered an advantage in developing a non-invasive method for EBV genomic DNA detection.

In current study, we pointed out the lower frequency of positive EBV cases among patients with NPC were 46.32%, 49.47%, 45.26% and 47.37% for *EBNA-1*, EBNA-2, LMP-1 and LMP-2, respectively, when compared to the previous study (Hao et al., 2004; Yap et al., 2007). However, such difference was statistically significant, compared to the non-cancerous samples ($p < 0.001$). It could be explained that the geographic variation in the distribution and prevalence of EBV. Moreover, The OR and RR were 81.10 ($p < 0.0001$) and 44.00 ($p = 0.0002$) for *EBNA-1*, 187.06 ($p = 0.0003$) and 95.00 ($p = 0.0013$) for EBNA-2, 158.26 ($p = 0.0004$) and 87.00 ($p = 0.0016$) for LMP-1, and 84.60 ($p < 0.0001$) and 45.00 ($p = 0.0001$) for LMP-2, respectively. It meant that the detection of candidate genes was strongly correlated with nasopharyngeal cancer via the OR and RR with the significant statistic. Based on the OR, the odds for positive *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* in nasopharyngeal cancer were 81.10, 187.06, 158.26 and 84.60 times higher than in the case without *EBNA-1*, *EBNA-2*, *LMP-1*, *LMP-2* detection, which could be inferred that without EBV infected. More, the positive of *EBNA-1*, *EBNA-2*, *LMP-1* and/or *LMP-2* was 44, 95, 87 and 45 times higher than negative in nasopharyngeal cancer. Based on those results, the detection of *EBNA-1*, *EBNA-2*, *LMP-1*, *LMP-2*, which indicated the infection of EBV, could be served as the potential biomarker for detection of EBV presence and predictor for nasopharyngeal cancer development and significant associated to risk of nasopharyngeal cancer development.

The combination of *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2*, was the most suitable for the diagnosis of NPC in nasopharyngeal brushing samples. It showed an advantage in detection of EBV genomic DNA within $PI \geq 0.25$, in that the frequency was increased to 57.89% which was higher than the frequencies of individual gene detection. It notably marked that in the case of screening on total of screening four genes, the ability to detect the present of EBV genomic DNA was very high, indicated the pre-requisite step in nasopharyngeal tumorigenesis. Moreover, of note is the four genes combined in screening and diagnosis has the specificity value of 57.89%, the sensitivity value of 97.89, Positive predictive value of 96.49% and Negative predictive value of 69.92%. Thus, it indicated the PCR-based EBV detection by four genes combined could be considered to be superior to the single-gene detection of each individual gene. Since the detection of multiple genes could guide the clinician in prognosis, diagnosis and taking a caution in the risk of NPC development in cases of unknown primary clinicopathological criteria.

In conclusion, *EBNA-1*, *EBNA-2*, *LMP-1* and *LMP-2* detection in nasopharyngeal samples obtained in 46.32%, 49.47% and 45.26%, and 47.37%, respectively. Notably, low frequency of those genes were observed in non-cancerous samples. Additionally, the significant correlation between was found between in the presence of these candidate genes and nasopharyngeal carcinoma as well as the odds ratio and relative risk were found in the significant association. Combining all genes in detection, it showed higher frequency with $PI \geq 0.25$,

counting for 57.89%, which was higher than detection based on individual gene. The candidate genes detection in nasopharyngeal brushing samples offered a chance in development of easily, rapid method for diagnosing NPC, in Vietnamese population. Notably, in Vietnam, there was still limit research related to evaluate prevalence of EBV genomic DNA, thus, this approach was directly demonstrated to evaluate developed standard PCR method in detection of these candidate genes in Vietnamese nasopharyngeal patients.

Conflicts of interest

The authors declared that they have no competing interests.

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