International Journal of Biomedical Science & Bioinformatics
Volume 3 : Issue 2 *[ISSN 2475 - 2290]*
Publication Date : 31 August, 2016

Non-invasive detection of *LMP-1*, *LMP-2* (Epstein-Barr Latent membrane protein) load in the diagnosis of nasopharyngeal carcinoma in Vietnamese population based on nasopharyngeal brushing samples

Lao Duc Thuan, Nguyen Hoang Anh Tuan, Luong Bao Duy, Tran Ngoc Quynh, Bui Thi Duong Anh, Ngo Dong Kha, Ho Ta Giap, Nguyen Hai Chau, Nguyen Huu Dung, Le Huyen Ai Thuy*

***Abstract* – In Vietnam, there have been reported to be highly incident cancer of nasopharynx. Epstein-Barr virus (EBV) have been considered to be mainly associated with nasopharyngeal carcinoma (NPC), in which EBV-encoded latent protein 1, 2 (*LMP-1*, *LMP2*) were found to be important factor target to NPC development. Thus, it is important to explore whether a non-invasive method could be applicable to screen and early diagnosis of NPC. In current study, NP (nasopharyngeal) and non-NP brushing samples were collected consecutively from participants when they underwent NP biopsy at Cho Ray Hospital, HCMC, Vietnam. PCR assay were used to detection of *LMP-1*, *LMP-2* and T-test statistical analysis were used to analyze its diagnosis value. NP-brushing samples from NPC patients showed frequency of 45.00% and 60.00% for *LMP-1* and *LMP-2*, respectively. Conversely, none of any cases of non-NP brushing samples were found to be positive to target both/individual genes. Virtually, in combination of both two genes, the frequency was increased to 66.67% as well as the PI value (PI ≥ 0.5). The *p* value < 0.05 (*LMP-1*: *p* = 0.037; *LMP-2*: *p* = 0.008; both: *p* = 0.004) was observed, that indicated the correlation of between the presence both *LMP-1*, *LMP-2* and NPC. The specific value was 100.00% for each/both candidate genes. Moreover, a high odd ratio (OR) and relative risk (RR) were also calculated of each/both candidate genes. In the case of *LMP-1*, OR, RR were 25.6087 (95%CI = 01.3479 - 486.545, p = 0.0309), 14.4762 (95%CI = 0.9085 - 230.672, p = 0.00585), respectively; in the case of *LMP-2*, the OR, RR were 45.5882 (95%CI = 2.391 – 869.201, p = 0.0111), 19.0476.11 (95%CI = 1.2166 – 298.2256, p = 0.0358), respectively; both candidate genes, the OR and RR were 58.8000 (95%CI = 2.9081 – 1070.6992, p = 0.0076), 26.5714 (95%CI = 1.3193 – 320.7527, p = 0.0309), respectively. Due to those results, the detection of both *LMP-1* and *LMP-2* in NP brushing samples could be an effective supplement for NPC early diagnosis that being non-invasive and rapid, demonstrated great potential for screening the high-risk of NPC in Vietnamese population.**

***Keywords:* non-invasive, *LMP-1*, *LMP-2*, nasopharyngeal carcinoma, NP brushing sample, Vietnamese population.**

[1]Lao Duc Thuan, Nguyen Hoang Anh Tuan, Luong Bao Duy
University of Science, Vietnam National University
Ho Chi Minh City, Vietnam.
[2]Lao Duc Thuan, Ngo Dong Kha, Ho Ta Giap, Nguyen Hai Chau, Le Huyen Ai Thuy
Ho Chi Minh City Open University
Ho Chi Minh City, Vietnam
[3]Tran Ngoc Quynh, Bui Thi Duong Anh, Nguyen Huu Dung
Cho Ray Hospital
Ho Chi Minh City, Vietnam

I. Introduction

Nasopharyngeal carcinoma has considered remarkably distinctive geographic and ethnic contribution, gravitating toward Southeast Asia, especially in China and Vietnam. In Vietnam, high prevalence of NPC cases was observed in reached to 4,931 cases (ASR = 5.4/100,000) and deaths was 2,885 cases (ASR = 3.3/100,000) (Globocan, 2012). Unfortunately, because of the ambiguous symptoms, such as hearing loss, nosebleeds, headache, etc. thus, most of NPC patients are only diagnosed when the tumor has reached an advanced stage [3, 7, 8]. Thus, the early diagnosis for NPC is essential in achieving a satisfactory therapy effect [10]. As NPC tumor, Epstein-Barr virus (EBV) infection, one of the high-risk factors leading to NPC, has been recognized to be strongly linked to NPC. EBV infection was shown to be early event and present in many copies and pre-invasive stage of NPC [6, 7, 9]. Therefore, the detection of genomic DNA of EBV may be highly predictive of NPC. Numerous studies have been performed have evaluated the presence of genomic DNA based on various methods, such as PCR, in situ hybridization, real-time PCR, etc. In which *LMP-1* (Latent membrane protein-1), *LMP-2* (Latent membrane protein-2) had proved that to be useful target oncogenes to screen NPC [7, 9]. As the function, *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 [1, 7]. The function of *LMP-2* is considered as playing important role in carcinogenesis by driving EBV into latency [1, 5]. In Vietnam, the study of combination of multiple genes in detection, especially in combination of *LMP-1*, *LMP-2* in detection, was still no research carried out in Vietnamese population. Therefore, the aim of the present study was to systematically evaluate the presence of those both *LMP-1* and *LMP-2* in Vietnamese nasopharyngeal brushing samples by PCR, for development of a non-invasive method for early diagnosis of NPC in Vietnamese population.

II. Materials and methods

A. Samples collection, DNA extraction

From June, 2015 to December, 2015, 20 nasopharyngeal bushing (nasopharyngeal swab) samples were retrieved from Cho Ray Hospital, Vietnam. All of those biopsies were collected from patients, which was obeyed to ethical approval for study human sample, and patients agreed with purpose of the study. All of samples were submitted to histopathological department to confirm NPC. Additionally, 10 nasopharyngeal swab samples were collected from healthy volunteers were used as negative controls.

DNA was extracted from clinical sample by means of an enzyme digestion using 700 µl lysis buffer (NaCl 5M, Tris-HCl 1M, EDTA 0.5 M, SDS 10% and Proteinase K 1 mg/ml). The samples were incubated at 56^{o}C overnight. Then, DNA obtained and purified by Phenol/Chloroform extraction and ethanol precipitation. The quality and purity of DNA extraction was measured by the proportion of A260/A280. Then, the DNA solution was stored at EDTA 0.5M, -20^{o}C for further used.

B. Detection of LMP-1, LMP-2

Viral DNA detection was carried out by PCR method for amplification of *LMP-1* and *LMP-2*. The forward and reverse primer sequences for *LMP-1* amplification were, respectively, 5'-CAGTCAGGCAAGCCTATG-3' and 5'-CTGCTTCCGGTGGAGATG-3' [4]. The forward and reverse primer sequences for *LMP-2* amplification were 5'-AGCTGTAACTGTGGTTTCCATGAC-3' and 5'-GCCCCCTGGCGAAGAG-3', respectively [4]. For PCR assay, the amplification was done in a total volume of 15 µl, containing 1 µg DNA template. PCR reaction was subjected to initial at 95^{o}C for 5 minutes, followed by 35 cycles at 95^{o}C for 30 seconds, X^{o}C for 30 seconds (Note: X = 65.5^{o}C, 65.0^{o}C for *LMP-1* and *LMP-2*, respectively), 72^{o}C for 30 seconds, and finally 72^{o}C for 10 minutes. Each PCR product was directly loaded onto a 2.0% agarose gel, stained with Ethidium bromide, and directly visualized under UV illumination. Then, PCR product was sequenced to confirm target gene amplification.

C. Statistical analysis

The statistical analysis was done by using the Medcalc® software. The frequencies of *LMP-1* and *LMP-2* were calculated and the Chi-squared test was used to compare the frequencies of categorical variables between groups. The differences of frequencies of *LMP-1*, *LMP-2* among groups were considered statistically significant for $p \leq 0.05$.

D. Results and discussion

Due to unclear symptoms, nasopharyngeal cancer often presents in the last stage when first diagnosis. Therefore, there is a challenge for finding a simple and non-invasive methods access to NPC. In current study, nasopharyngeal brushing samples have been enrolled for the non-invasive diagnosis of NPC. According to Chang et al. (2003), Zhang et al. (2012), among a variety of non-invasive samples, such as serum, plasma, NPC swab, etc. NPC swab achieved the highest sensitivity and specificity in quantity and quality, since it gathers tumor cells by contacting the primary tumor directly, and other advantages such as eases of application and swiftness. Thus, these advantages make the NP swab extremely amenable for non-invasive method screening purposes.

In present study, PCR method was applied in evaluation of two target genes, *LMP-1* and *LMP-2* in NPC brushing samples and healthy samples (Table I). As the results, the frequency of *LMP-1*, *LMP-2* detection were 45.00%, 60.00% in NP brushing samples, respectively, significantly, in healthy controls, total of healthy specimens were negative to *LMP-1* and *LMP-2*. The PCR products were observed by electrophoresis in distinctly different sizes and easily identified. The *LMP-1* forward and reverse primer yielded a PCR product of 106 bp, whereas the *LMP-2* PCR was 69 bp (Fig. 1). Furthermore, PCR product was confirmed by DNA sequencing. The signal of peaks in PCR product sequencing was quite good for reading nucleotide (Data not shown). According to Blast results, *LMP-1* and *LMP-2* sequence were similar to Human Herpesvirus 4 (Epstein-Barr virus) 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*). As shown in Table 1, Chi-squared test was applied to evaluate the correlation between target genes and NPC, calculated by Medcalc® software, it indicated that, in our study, the presence of *LMP-1* or *LMP-2* was strongly correlated with nasopharyngeal carcinoma (*LMP-1*: $p = 0.0087$; *LMP-2*: $p = 0.0008$). It was according to previous studies, suggested that the presence of EBV genomic, in this status, *LMP-1* and *LMP-2*, had proved that to be useful target oncogenes to screen NPC [7, 9].

TABLE I. VALIDITY DATA FOR *LMP-1* AND *LMP-2* GENE DETECTION IN SAMPLE COLLECTION

	LMP-1		***LMP-2***	
	Positive n(%)	***Negative n(%)***	***Positive n(%)***	***Negative n(%)***
Nasopharyngeal Carcinoma (n = 31)	9 (45.00)	12 (55.00)	12 (60.00)	8 (40.00)
Healthy specimens (n = 10)	0 (0.00)	10 (100.00)	0 (0.00)	10 (100.00)
p value	0.0087		0.0008	

Additionally, the risk of nasopharyngeal cancer was also calculated via odds ratio (OR) and relative risk (RR) by analysis of detection of *LMP-1*, *LMP-2* in samples. Results indicated that, in the case of *LMP-1*, OR = 25.6087 (95% CI = 1.3479 - 486.545, $p = 0.0309$) and the RR = 14.4762 (95% CI = 0.9085 - 230.672, $p = 0.00585$); in the case of *LMP-2*, OR = 45.5882 (95% CI = 2.391 – 869.201, $p = 0.0111$) and RR = 19.0476.11 (95% CI = 1.2166 – 298.2256, $p = 0.0358$). Therefore, it could be induced that the presence of *LMP-1*, *LMP-2* were significant features of nasopharyngeal carcinoma, in Vietnamese population.

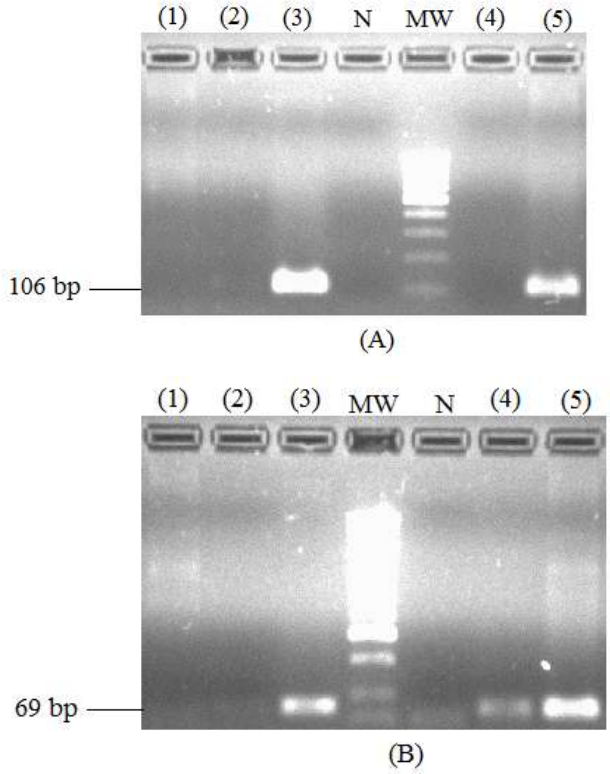

Figure 1. Agarose gel electrophoresis showing the present of (A) *LMP-1*, (B) *LMP-2. NW: Molecular ladder (A) 100 bp, (B) 50 bp. (1) (2): Healthy samples; (3) (4) (5): Nasopharyngeal brushing samples.*

In screening both two genes, pairwise combination of *LMP-1* and *LMP-2*, it showed an advantage in specific detection of EBV in nasopharyngeal cancer in Vietnamese patients via PI (PCR index) ≥ 0.5, which meant that at least one of *LMP-1* or *LMP-2* was detection, counting for 65.00% (Table II). It was higher than detection based on individual gene as 45.00% for *LMP-1* and 60.00% for *LMP-2*. The *p* value = 0.0003 also showed the correlation between detection of at least one gene and NPC. It indicated the detection of combination of both genes leading to the sensitive appearance of *LMP-1* and *LMP-2* in nasopharyngeal brushing samples in which may be explained by the presence of EBV in NPC. Moreover, the OR = 58.8000 (95%CI = 2.9081 – 1070.6992, $p = 0.0076$) and RR = 26.5714 (95%CI = 1.3193 – 320.7527, $p = 0.0309$) were calculated. Thus, the results of current study indicated that the detection of both *LMP-1* and *LMP-2* in NP brushing samples, especially in combination both genes, could be an effective supplement for NPC early diagnosis that being non-invasive and rapid, demonstrated great potential for screening the high-risk of NPC in Vietnamese population.

TABLE II. THE PI (PCR INDEX) OF *LMP-1* AND *LMP-2* GENE DETECTION IN SAMPLE COLLECTION

PI	n (%)
0 (none gene was detected)	7 (35.00)
0.5 (one gene was detected)	2 (10.00)
1.0 (both genes were detected)	11 (55.00)
≥ 0.5 (At least one gene detected)	13 (65.00)

III. Conclusion

In summary, a non-invasive method was developed, which could be applied in detection of the *LMP-1* and *LMP-2* presence, based on nasopharyngeal brushing samples. In current study, the frequency of *LMP-1* and *LMP-2* presence were 45.00%, 60.00%, respectively. In healthy samples, no any sample was positive to *LMP-1* or *LMP-2*. We demonstrated that, in two target genes combined, the frequency of at least one gene detected (PI ≥ 0.5) reached to 65.00%, and the OR, RR were 58.8000, 26.5714, respectively. Therefore, these finding suggested that, based on the detection of candidate genes in nasopharyngeal brushing samples, was easy to be handled for non-invasive screening method.

Acknowledgement

This research was conducted on the support of the Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam.

References

[1] C. W. Dawson, R. J. Port, and L. S. Young LS, "The role of the EBV-encoded latent membrane proteins LMP1 and LMP2 in the pathogenesis of nasopharyngeal carcinoma (NPC)", Semin Cancer Biol., vol 22, pp. 144-153, April 2012.

[2] H. W. Chang ,A. Chan, D. L. Kwong, W. I. Wei, J. S. Sham, and A. P. Yuen, "Evaluation of hypermethylated tumor suppressor genes as tumor markers in mouth and throat rinsing fluid, nasopharyngeal swab and peripheral blood of nasopharygeal carcinoma patient", Int J Cancer., vol 105, pp. 851-855, July 2003.

[3] J. B. Epstein and C. K. Jones, "Presenting signs and symptoms of nasopharyngeal carcinoma", Oral Surg Oral Med Oral Pathol., vol 75, pp. 32-36, January 1993.

[4] J. L. Ryan, H. Fan, S. L. Glaser, S. A. Schichman, N. Raab-Traub, and M. L. Gulley, "Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments: a novel approach to screen for the virus in paraffin-embedded tissue and plasma", J Mol Diagn., vol 6, pp. 378-385, November 2004.

[5] M. P. Thompson and R. Kurzrock, "Epstein-Barr virus and cancer", Clin Cancer Res., vol 10, pp. 803-821, February 2004.

[6] R. Pathmanathan, U. Prasad, R. Sadler, K. Flynn, and N. Raab-Traub, "Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma", N Engl J Med., vol 333, pp. 693-698, September 1995.

[7] S. P. Hao, N. M. Tsang, K. P. Chang, and S. H. Ueng, "Molecular diagnosis of nasopharyngeal carcinoma: detecting *LMP-1* and EBNA

by nasopharyngeal swab", Otolaryngol Head Neck Surg, vol 131, pp. 651-654, November 2004.

[8] W. I. Wei and D. L. Kwong, "Current management strategy of nasopharyngeal carcinoma", Clin Exp Otorhinolaryngol., vol 3, pp. 1-12, March 2010.

[9] Y. Y. Yap, S. Hassan, M. Chan, P. K. Choo, and M. Ravichandran, "Epstein-Barr virus DNA detection in the diagnosis of nasopharyngeal carcinoma", Otolaryngol Head Neck Surg., vol 136, pp. 986-991, June 2007.

[10] Z. Zhang, D. Sun, S. H Hutajulu, I. Nawaz, Nguyen Van D, G. Huang, S. M. Haryana, J. M. Middeldorp, I. Ernberg, and L. F. Hu, "Development of a non-invasive method, multiplex methylation specific PCR (MMSP), for early diagnosis of nasopharyngeal carcinoma", PLoS One., vol 7, pp. e45908, 2012.

About author (s):

Le Huyen Ai Thuy, PhD, Associate Professor.
Research field: Molecular oncology, Cancer epigenetics, etc.

Lao Duc Thuan, MSc, PhD student.
Research field: Molecular of Cancer, Cancer epigenetics, etc.