EVALUATION OF *EBNA-1* (EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN-1) GENE PREVALENCE IN NASOPHARYNGEAL CARCINOMA IN VIETNAMESE PATIENTS

Lao Duc Thuan[1,2], Nguyen Hoang Anh Tuan[1], Ngo Dong Kha[2], Thieu Hong Hue[2], Ho Ta Giap[2], Nguyen Hai Chau[2], Nguyen Huu Dung[3], Le Huyen Ai Thuy[2,*]

[1]University of Science, Vietnam National University Ho Chi Minh City, Vietnam.

[2]Ho Chi Minh City Open University, Vietnam.

[3]Cho Ray Hospital, Ho Chi Minh City, Vietnam.

*Email: thuy.lha@ou.edu.vn

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ABSTRACT

This study examined the presence of Epstein-Barr virus (EBV) in nasopharyngeal carcinoma (NPC) based on the detection of EBNA-1 (Epstein-Barr virus nuclear antigen-1) by Polymerase Chain Reaction (PCR), in Vietnamese population. Firstly, we systematically analyzed the mean of percentage weighted of the presence of EBNA-1 in previous relevant studies. Experimentally, 31 nasopharyngeal cancer biopsies and 20 healthy samples were enrolled in current to evaluate the frequency of candidate genes. As the results, the frequency of EBNA-1 was 77.42%, whereas, none of any cases of healthy samples were found to positive to target gene. The p value < 0.05 (p = 0.0001) showed that it was significant correlation between the presence of this candidate gene and nasopharyngeal cancer. Moreover, a high odds ratio (OR) and relative risk (RR) of candidate gene, (OR = 68.16, RR = 2.41) were calculated. Therefore, the detection of EBNA-1, which performed by PCR, could serve as a good supplement to early diagnosis and prognosis of NPC in Vietnamese population.

Keywords: *EBNA-1; Nasopharyngeal carcinoma; Vietnamese population.*

1. Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and malignant tumor of the nasopharynx, the uppermost region of the pharynx. NPC has a striking and geographic and ethnic distribution, which encountered in Asian region, gravitating towards Southeast Asia, especially in China and Vietnam (da Costa *et al*, 2015; Pathmanathan *et al*, 1995). 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 were 4,931 cases (ASR = 5.4/100,000) and deaths was 2,885 cases (ASR = 3.3/100,000) (GLOBOCAN, 2012). The etiology of NPC is strongly suggested to be involved in several genetic susceptibilities and environmental factors. Notably, within ambiguous symptoms, such as hearing loss, nosebleeds, headache, trouble opening the mouth, etc., thus, most patients present with the stage III or IV cancer when diagnosed (Epstein, Jones, 1993; Hao *et al*, 2004), thus, it could be a challenge in treatment. Therefore, to achieve favorable treatment and increasing to patient's

survival, early diagnosis and prognosis are necessary to be appropriate managed. To date, three major etiological factors, including viral infection, genetic and environment factors, are defined (Frappier, 2012; Lung *et al*, 2014; Lo *et al*, 2004). Many studies have proved that NPC is strongly linked to Epstein-Barr virus (EBV) infection, which is a gamma herpes virus discovered in 1964 by Epstein and Barr, taxonomically called Human herpes virus 4 (Frappier, 2012; Lung *et al*, 2014; Epstein *et al*, 1964; Chan, Wong, 2014). EBV infection was considered to be an early event, pre-requisite step in nasopharyngeal tumorigenesis and may contribute to its pathogenesis (Chan, Wong, 2014; Yip *et al*, 2010). Since then, viral infection has been received much attention and represented a prospective biomarker for the prognosis and diagnosis of NPC. For the past decade, owing to the increase in EBV 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 various sources of NPC. Moreover, many previous studies indicated the prevalence of EBV attributed to NPC was reported variations in different ages, sex, geographic regions (Chang, Adami, 2006; Khan, Hashim, 2014). For instance, in the high incidence regions, such as East Asia, South Asia, South East Asia and North Africa and Middle East, etc., within the prevalence were up to 100% (Chang, Adami, 2006; Khan, Hashim, 2014), nevertheless, the overall EBV sero-prevalence in samples from North and South China were 80.78% and 79.38%, respectively (Xiong *et al*, 2014).

EBV consists a double-stranded 184-kbp-long DNA enclosed in a protein capsid and that encodes more than 85 genes. Evidences showed that multiple copies of EBV genome are presented nasopharyngeal carcinoma and EBV DNA is also found in the pre-invasive state of NPC (Pathmanathan *et al*, 1995; Yap *et al*, 2007). The EBV genomic organization that code for EBNAs (Epstein-Barr virus nuclear antigens) including *EBNA-1*, *2*, *3A*, *3B*, *3*C and *LP*; *LMP-1*, *2A* and *2B* (latent membrane protein); two small *EBERs* (non-coding nuclear RNAs) (Hammerschmidt, Sugden, 2013; Young *et al*, 1988). Individually, *EBNA-1* plays an important role of EBV infection. As function, EBNA-1 is essential for EBV immortalization of the 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).

The study of evaluation prevalence of EBV, by detection of *EBNA-1*, was still no research carried out in Vietnamese population. Whereas, only research was carried out in detection of the EBV genetic variation on *LMP1* (Nguyen-Van D *et al*, 2008) and MMSP (multiplex methylation PCR) (Zhang *et al*, 2012) for Vietnamese patients with nasopharyngeal carcinoma. Therein, the aim at the present study was to systematically evaluate the mean of *EBNA-1* based on previous studies and to analyze the presence of this gene in Vietnamese nasopharyngeal carcinoma tissues by PCR.

2. Materials and methods

Data mining, the identification of presence of *EBNA-1* in previous relevant studies

The following keywords: Epstein-Barr virus, nasopharyngeal carcinoma, *EBNA-1*, etc., were applied to identification of detection of EBV genomic DNA for early diagnosis and prognosis of nasopharyngeal cancer. Moreover, the following databases were

searching: PubMed, ScienceDirect, etc. The selection period lasted until the end of 2015. Consequently, the data onto *EBNA-1* frequencies were extracted and used to calculate of means of their presences.

Samples collection, DNA isolation

From June, 2015 to December, 2015, thirsty one nasopharyngeal tumor biopsies (Male: 74.19% and female: 25.81%) were retrieved from Cho Ray Hospital, Vietnam. All of those nasopharyngeal biopsies were submitted to histopathological department to confirm NPC. Additionally, twenty nasopharyngeal swab samples were collected from healthy volunteers were used as negative controls. DNA was extracted from clinical samples by means of an enzyme digestion using 700 µl lysis buffer (NaCl 5M, Tris-HCl 1M, EDTA 0.5M, 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 A_{260}/A_{280}. Then, the DNA solution was stored at EDTA 0.5M, -20^{o}C for further used.

Detection of *EBNA-1*

Viral DNA detection was carried out by PCR method to *EBNA-1* gene amplification. The forward and reverse primer sequences were: 5'-GTCATCATCATCCGGGTCTC-3' and 5'-TTCGGGTTGGAACCTCCTTG-3', respectively (Telenti *et al*, 1990). Amplification of human beta-actin gene was used as internal control for the marker of the presence for intact genomic DNA. The forward and reverse primer sequence to beta-actin amplification were: 5'- ATCATGTTTGAGACCTTCAACAC-3' and 5'- CATCTCTTGCTCGAAGTCCAG-3', respectively.

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, 63^{o}C for 30 seconds, 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.

Statistical analysis

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

3. Results

Data mining, presence of *EBNA-1* in previous studies

The criteria for selection related research articles in database were the selection period lasted until 2015 within *EBNA-1* detection on various samples, such as nasopharyngeal carcinoma tissues, nasopharyngeal swab, etc., by different methods as PCR, RT-PCR, immunohistochemistry, etc., as the result, total of 16 relevant studies was found. As the results, the detection of target gene in nasopharyngeal cancer cells from various sources such as biopsy tissue, serum, nasopharyngeal swab, peripheral blood, etc. and carried out on various countries, such as India, Malaysia, Iran, etc. However, the predominant kind of sample was biopsy tissue. For target gene detection, among several methods used, such as PCR, RT-PCR, nested-PCR, etc., PCR was the most common method for detection of *EBNA-1*. The frequency of *EBNA-1* was ranged from 65.0% to 100%. The estimated frequencies of *EBNA-1* variants of the samples of NPC patients were necessary to be calculated, counting for 93.50%. Additionally, the mean of detection of *EBNA-1* in non-cancer samples, including various types as tissues, blood, swab or paraffin-embedded tissues, were 13.8%, respectively, shown in Fig. 1.

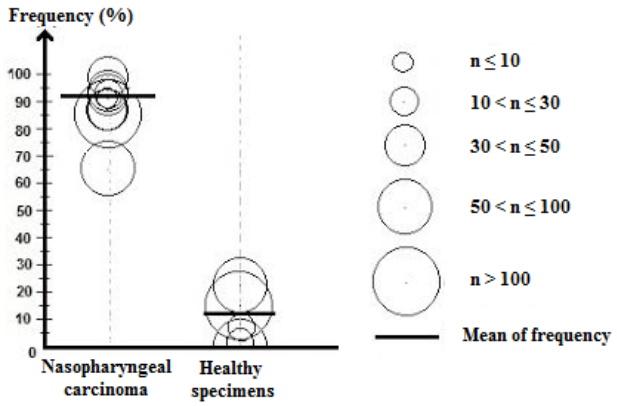

Figure 1. The frequency of *EBNA-1* in Nasopharyngeal cancer and healthy specimens in previous studies. The center of the circle indicated the previous reported frequencies and the size of the circle indicated the size of studies (n = number of samples), number of the circle indicated amount of previous studies.

The frequencies of *EBNA-1* in nasopharyngeal cancer specimens and healthy samples

Total samples were enrolled in PCR for detection of *EBNA-1* prevalence. The frequency of *EBNA-1* was evaluated by PCR assay including nasopharyngeal cancer biopsy specimens and healthy specimens. As well as κ-value, sensitivity, specificity, positive and negative predictive values, likelihood ratio and receiver-operating characteristic were shown in Table 1. As the results, of the 31 NPC tumors, the frequency of *EBNA-1* detection was 77.42% (24 of 31 cases). Concerning to healthy specimens, total of healthy specimens were negative to *EBNA-1* detection.

Table 1. Validity data for individual gene detection in nasopharyngeal cancer specimens and healthy samples

		EBNA-1
Nasopharyngeal specimens	P (n(%))	24 (77.42)
	N (n(%))	7 (22.58)
Healthy specimens	P (n(%))	0 (0.00)
	N (n(%))	20 (100.00)
	κ	0.729
	AUC	0.887
	Se	77.42%
	Sp	100.00%
	Pp	100.00%
	Np	74.07%
	LR-	0.23
	p-value	*< 0.0001*

Note: P: positive; N: negative; κ: Kappa value; Se: Sensitivity; Sp: Specificity; Pp: Positive Predictive Value; Np: Negative predictive value; LR-: Negative Likelihood Ratio; AUC: Area under the ROC curve.

The PCR products were observed by electrophoresis in distinctly different sizes and easily identified, shown in Fig. 2. The *EBNA-1*, forward and reverse primer yielded a PCR product of 269 bps. For internal control, the *beta-actin* PCR was 319 bps. Furthermore, PCR product was confirmed by DNA sequencing. The signal of peaks of PCR product sequencing were good for reading nucleotide (Fig. 3). According to Blast results, *EBNA-1* gene's sequence was similar to Human Herpesvirus 4 (Epstein-Barr virus). *EBNA-1* sequence was similar to KP735248, within Total score = 462, Ident = 100% and E-value = 1e-126.

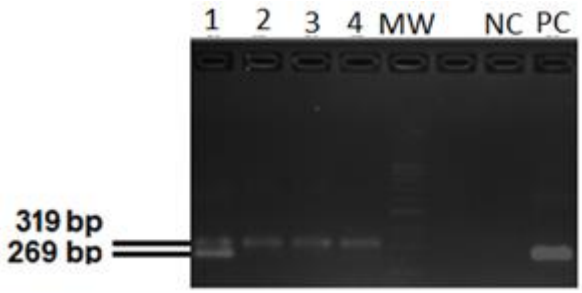

Figure 2. Agarose gel electrophoresis showing the present of *EBNA-1* and *beta-actin* in representative samples. (1)(2): NPC biopsy samples; (3)(4) Healthy samples; NC: Negative control; PC: positive control. MW: molecular weight ladder 100 bp.

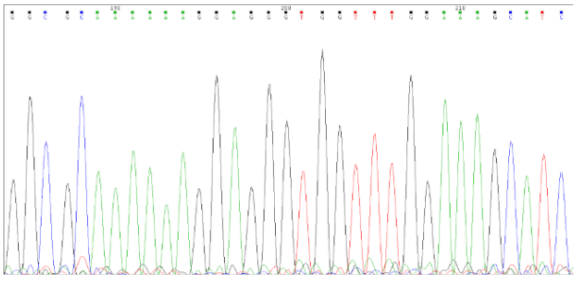

Figure 3. Sequencing profile of *EBNA-1* of represent nasopharyngeal sample.

Chi-squared test was used to access the correlation between candidate genes and nasopharyngeal carcinoma was calculated by Medcalc® software, it indicated that, in our study, the presence of *EBNA-1* was strongly correlated with nasopharyngeal carcinoma (p = 0.0001). Additionally, the risk of nasopharyngeal cancer was also calculated via odds ratio (OR) and relative risk (RR) by analysis of detection of *EBNA-1* in samples. Results indicated that, in the case of *EBNA-1*, OR = 68.16 (95% CI = 3.58 - 1314.13, p = 0.0050) and the RR = 2.41 (95% CI = 1.37 – 4.27, p = 0.0022). Therefore, it could be induced that the presence of *EBNA-1* was significant feature of nasopharyngeal carcinoma, in Vietnamese population.

4. Discussion

Due to unclear symptoms, nasopharyngeal cancer often presents in the last stage when first diagnosis. Therefore, there is a challenge to finding a simple and non-invasive methods access to NPC. In current study, the data mining provided the evidence that presence of *EBNA-1* was a striking characteristics of genomic EBV contributed to nasopharyngeal cancer. In our data mining, the range of frequency of *EBNA-1* was ranged from 65.0% to 100%. These results were in accordance with previous statements that prevalence of the presence of *EBNA-1* was various in different countries (da Costa *et al*, 2015; Pathmanathan *et al*, 1995). As shown in figure 1, the mean of presence of *EBNA-1* gene was 93.50%. Whereas, total healthy samples were found of 13.8%. Therefore, it indicated that the presence of *EBNA-1* could be considered as the potential molecular biomarker in early diagnosis of NPC. In Vietnam, there was still no research related to evaluate prevalence of EBV, only research was carried out in evaluation of *LMP1* (Nguyen-Van D *et al*, 2008) and MMSP (multiplex methylation PCR) (Zhang *et al*, 2012) for Vietnamese patients with nasopharyngeal carcinoma. According to Zhang *et al* (2012), an ideal cancer screening and detection test should be non-invasive to be performed, however, in our study, the biopsy tissues were enrolled, because biopsy

samples are highly accurate since these samples were obtained from nasopharyngeal tumors. In our study, in the nasopharyngeal cancer biopsy set, the frequency of *EBNA-1* was 77.42%, which was lower than the mean of this gene in our data mining. In the healthy control, it was according to previous studies that lower than nasopharyngeal carcinoma, especially no positive case was found. Based on figure 2, the PCR product was easily distinguished from the different length, among them, the human *beta-actin* and positive control for *EBNA-1* gave indication on the quality of the extracted DNA and specific of primer sets used. According to table 2, a value $p < 0.05$ (p = 0.0001) showed a strong correlation between the presence of each *EBNA-1* with nasopharyngeal carcinoma, inferred the presence of EBV, were significant associated with nasopharyngeal cancer development. The OR and RR were 68.16 (p = 0.0050) and 2.41 (p = 0.0022) for *EBNA-1*. It meant that the detection of candidate gene 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* in nasopharyngeal cancer was 68.16 times higher than in the case of negative *EBNA-1* detection, which meant that without EBV infected. More, the positive of *EBNA-1* were 2.41 times higher than negative in nasopharyngeal cancer. Based on those results, the detection of *EBNA-1* which indicated the infection of EBV, could be served as the potential biomarker for detection of EBV presence and predictor for nasopharyngeal cancer development. Moreover, specificity and positive predictive value, which reach to 100% and other values, including the highest sensitivity, κ-value, area under the receiver-operating characteristic curve in nasopharyngeal biopsy samples, making it suitable and specific gene for diagnosis of NPC.

5. Conclusion

During the data mining, total of studies was enrolled to evaluate mean of presence of candidate gene, which was a striking geographic and ethnic distribution of NPC cases, within the mean of *EBNA-1* was 93.50%. In our study, *EBNA-1* detection in nasopharyngeal biopsy samples obtained in 77.42%. Notably, none of the heathy control was positive. Moreover, the p value showed the strong correlation between the detection of *EBNA-1* and NPC. Additionally, the OR and RR were calculated, counting for 68.16 (p = 0.0050) and 2.41 (p = 0.0022), respectively. Therefore, the candidate gene detection in tissue obtained from nasopharyngeal biopsy suggested that PCR assay done in candidate gene on the patients' samples, which was easily, rapid for diagnosing NPC, in Vietnamese population. In future studies, non-invasive samples will be continuously carried out to find out the non-invasive method in early diagnosis and prognosis for nasopharyngeal cancer in Vietnamese population.

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REFERENCES

Chan, J. Y., Wong, S. T. (2014). The role of plasma Epstein-Barr virus DNA in the management of recurrent nasopharyngeal carcinoma, *Laryngoscope*, 124(1), 126-130.

Chang, E. T., Adami, H. O. (2006). The enigmatic epidemiology of nasopharyngeal carcinoma, *Cancer Epidemiol Biomarkers Prev.*, 15, 1765-1777.

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.

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

Epstein, J. B., Jones, C. K. (1993). Presenting signs and symptoms of nasopharyngeal carcinoma, *Oral Surg Oral Med Oral Pathol.*, 75, 32-36.

Epstein, M. A., Achong, B. G., Barr, Y. M. (1964). Virus particles in cultured lymphoblasts from Burkitt's lymphoma, *Lancet*, 1, 702-703.

Frappier, L. (2012). Role of EBNA1 in NPC tumourigenesis, *Semin Cancer Biol*, 22, 154-161.

GLOBOCAN. Estimated cancer incidence, mortality and prevalence worldwide in 2012. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx.

Hammerschmidt, W., Sugden, B. (2013). Replication of Epstein-Barr viral DNA, *Cold Spring Harb Perspect Biol.*, 5, a013029.

Hao, S. P., Tsang, N. M., Chang, K. P., Ueng, S. H. (2004). Molecular diagnosis of nasopharyngeal carcinoma: detecting LMP-1 and EBNA by nasopharyngeal swab, *Otolaryngol Head Neck Surg.*, 131, 651-654.

Khan, G., Hashim, M. J. (2014). Global burden of deaths from Epstein-Barr virus attributable malignancies 1990-2010, *Infect Agent Cancer.*, 9, 38.

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

Lung, M. L., Cheung, A. K., Ko, J. M., Lung, H. L., Cheng, Y., Dai, W. (2014). The interplay of host genetic factors and Epstein-Barr virus in the development of nasopharyngeal carcinoma, *Chin J Cancer*, 33, 556-568.

Nguyen-Van, D., Ernberg, Phan-Thi Phi, P., Tran-Thi, C., Hu, L. (2008). Epstein-Barr virus genetic variation in Vietnamese patients with nasopharyngeal carcinoma: full-length analysis of LMP1, *Virus Genes*, 37, 273-281.

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

Ryan, J. L., Fan, H., Glaser, S. L., Schichman, S. A., Raab-Traub, N., Gulley, M. L. (2004). 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.*, 6, 378-385.

Sivachandran, N., Wang, X., Frappier, L. (2012). Functions of the Epstein-Barr virus EBNA1 protein in viral reactivation and lytic infection, *J Virol.*, 86, 6146-6158.

Telenti, A., Marshall, W. F., Smith, T. F. (1990). Detection of Epstein-Barr virus by polymerase chain reaction, *J Clin Microbiol.*, 28, 2187-2190.

Thompson, M. P., Kurzrock, R. (2004). Epstein-Barr virus and cancer, *Clin Cancer Res.*, 10, 803-821.

Xiong, G., Zhang, B., Huang, M. Y., Zhou, H., Chen, L. Z., Feng, Q. S., Luo, X., Lin, H. J., Zeng, Y. X. (2014). Epstein-Barr virus (EBV) infection in Chinese children: a retrospective study of age-specific prevalence, *PLoS One.*, 9, e99857.

Yap, Y. Y., Hassan, S., Chan, M., Choo, P. K., Ravichandran, M. (2007). Epstein-Barr virus DNA detection in the diagnosis of nasopharyngeal carcinoma, *Otolaryngol Head Neck Surg.*, 136, 986-991.

Yip, Y. L., Tsang, C. M., Deng, W., Cheung, P. Y., Jin, Y., Cheung, A. L., Lung, M. L., Tsao, S. W. (2010). Expression of Epstein-Barr virus-encoded LMP1 and hTERT extends the life span and immortalizes primary cultures of nasopharyngeal epithelial cells, *J Med Virol.*, 82(10), 1711-1723.

Young, L. S., Dawson, C. W., Clark, D., Rupani, H., Busson, P., Tursz, T., Johnson, A., Rickinson, A. B. (1988). Epstein-Barr virus gene expression in nasopharyngeal carcinoma, *J Gen Virol.*, 69, 1051-1065.

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