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The Expression of mRNA LMP1 Epstein-Barr Virus from FFPE Tumour Biopsy: a Potential Biomarker of Nasopharyngeal Carcinoma Diagnosis

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Abstract

Nasopharyngeal carcinoma (NPC) is a multifactorial disease that is endemic geographically in the world. Indonesian population has a highly incidence rate that is 6.2/100,000 people year. The pathogenesis of NPC is more directly reflected by carcinoma-specific viral transcriptional activity at the site of primary tumour. Epstein-Barr virus (EBV) infection in NPC is reflected by the expression of EBV latent and lytic gene. In fact, mRNA Latent Membrane Protein 1 (LMP1) EBV expression was an important latent infection biomarker. The aim of this study was to determine a potential use of relative expression of mRNA LMP1 EBV from formalin-fixed paraffin embedded (FFPE) tumour biopsy in NPC as a tumour biomarker. This reseach design was a cross sectional study. The samples were the archived specimens of FFPE tumour biopsy from NPC WHO-3 patient which were collected from untreated patients from 2014 in the Department of Pathology Anatomy, Prof. dr. Margono Soekarjo Hospital, Purwokerto. The expression of mRNA LMP1 EBV expression was determined by RT-PCR technique. The positivity of mRNA LMP1 EBV expression was 51.9%, indicating a moderate positivity. The result proved that the expression of mRNA LMP1 EBV from FFPE NPC WHO-3 tumour biopsy was a potential biomarker of NPC diagnosis. The molecular methods would improved the management of NPC, particularly in the histopathological diagnosis of NPC.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) has a significant difference in the geographical distribution and ethnicity (Yu & Yuan, 2012; Chang & Adami, 2006). NPC is endemic in certain regions of the world, especially in Southeast Asia, and has a poor prognosis. In Indonesia, the recorded mean prevalence is 6.2/100 000, with 13 000 yearly new NPC case (Soeripto, 1998; Adham et al., 2012). Etiology of NPC is multifactorial including host genetic factor, Epstein-Barr virus (EBV) infection and environmental factors. EBV infection has been shown to be consistent with the onset of NPC (Zur Hauzen, et al., 1970; Old et al., 1996; Roezin, 1999; Munir, 2006).

EBV has two phases in the cycle of infection, i.e., latent and lytic phase. In the latent phase, few EBV latent genes are expressed, so the number copies of viral DNA is maintained in a relatively low level and does not produce virions. In the lytic phase, a series of lytic gene is expressed caused by genome replication and virion production (Chang et al, 2004; Kieff & Rickinson 2001). EBV infection shows a pattern of latent infection phase II that will express Epstein-Barr virus Nuclear Antigen (EBNA-1) and Latent Membrane Protein I (LMP1). The direct measurement of activities mRNA EBV in primary tumour location on nasopharyngeal region needs to be done, because the activity of mRNA EBV reflects better virtually the pathogenesis of NPC than a serologic diagnosis and a measurement of EBV DNA in circulation (Steven et al., 2006). The advantages of using FFPE NPC tumour biopsy as samples were they was confirmed as NPC World Health Organization-3 (WHO-3) histopathologically and non invasive specimen. In prevoius study, the expression of mRNA LMP1 EBV genes have been proved only in fresh NPC tumour biopsy and is performed by Reverse Transcriptase Polymerase Chain Reaction technique (RT–PCR) (Wahyono et al, 2016). Reverse transcription process is a complement DNA (cDNA) synthesis by reverse transcriptase enzyme. PCR is an alternative method to identify the Avian Influenza (AI) virus, although the viral genomes in few quantities (Payungporn et al., 2004; OIE 2005; Wibowo et al., 2012). The research aimed at knowing potensial use for mRNA LMP1 expression from FFPE tumour biopsy in NPC WHO-3 as a biomarker of NPC diagnosis. Regarding to NPC management, it could be recommended to the clinician using a molecular technique, because the mRNA LMP1 expression method from FFPE tumour biopsy increased the accuracy of NPC diagnosis. It was the first study to analysis the mRNA expression of LMP1 EBV from FFPE NPC WHO-3 tumour biopsy as a tumour biomarker.

METHODS

This reseach design was a cross sectional study. The research was done from February 2015 until August 2015. All NPC formalin-fixed paraffin embedded (FFPE) tumour biopsies were collected from untreated patients who where histopathologically confirmed as NPC WHO-3 at Department of Pathology Anatomy, Prof. dr Margono Soekatjo Hospital/Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto. The histological diagnosis was confirmed by the pathologist involved in the study. Total of research subject were 27 people for untreated NPC WHO-3 patients from 2014. All research subjects were given informed consent before taking part in this research.

The 8-10 slices of NPC formalin-fixed paraffin embedded (FFPE) tumour biopsies were performed by PureLink FFPE RNA isolation kit protocol (Invitrogen) to obtain 50-100µL RNA solution. By 10 µL RNA solution, it could be directly used for the analysis of Reverse Transcriptase PCR (RT-PCR) or stored for long periods at a temperature of -80°C. cDNA was synthesis by cDNA Synthesis Super Script III First Strand Systemprotocol (Invitrogen) to obtain 20 µL cDNA solution.

Two steps of RT-PCR technique were performed to detect mRNA LMP1 EBV expression from FFPE tumour biopsy in NPCWHO-3 patients. A set of primer used to amplify the cDNA of LMP1 EBV gene resulted in a cDNA amplicon 142 bp consisting of a forward primer of 5'-GGAGATTCTCTGGCGACTTG-3' and a reverse primer of 5-GAGCCAAAGGAGATCAACCA-3'. Primer was designed by Primer3 software from the NCBI GenBank Sequence Database (accession number NC_009334.1, GeneID: 5176215). The composition of 15 µL was 7.5 µL Dream Taq PCR MasterMix – Thermo Scientific (Dream Taq DNA Polymerase, 2X Dream Taq Buffer, 4 mM $MgCl_2$, 0,4 mM dGTP, 0,4 mM d*ATP*, 0,4 mM dCTP, 0,4 mM dTTP). The PCR mix was runon a Thermocycler (Primus 25, PeqLab) by 35 cycles. The PCR condition of first step was pre-denaturation at 94°C for 5 min, denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, elongation at 72°C for 60 sec, and post- elongation at 72°C for 7 min. The amplicon was visualized by the electrophoresis on 2.5% agarose gel.

RESULTS AND DISCUSSION

Positivity expression of mRNA LMP1 EBV

Figure 1 shows the result of mRNA Latent Membrane Protein 1 (LMP1) Epstein-Barr Virus (EBV) from Formaline-Fixed Paraffin Embedded (FFPE) in Nasopharyngeal Carcinoma World Health Organization-3 (NPC WHO-3) tumour biopsy detected by RT-PCR technique on 2 % Agarose gel. Samples that expressed mRNA LMP1 EBV showed a 142 bp amplicon which represented on sample no. 1646, 713, 2822, 3028 and 2106. However, the sample no. 677 and 3274 did not express mRNA LMP1 EBV.

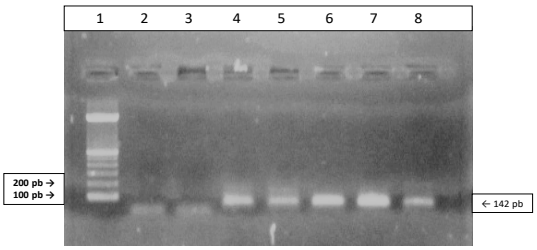

Figure 1. The results of mRNA LMP1 EBV from FFPE tumour biopsy in 3 NPC WHO-3 patients by RT-PCR technique as follow: no. 1. DNA marker (100 bp), no. 2 & 3 NPC WHO-3 patients who did not expressed mRNA LMP1 EBV (no. 677&3274), no.4-8NPC WHO-3 patients who expressed mRNA LMP1 EBV (no. 1646, 713,2822,3028 & 2106).

The positivity of mRNA LMP1 EBV was calculated by the proportion of NPC WHO-3 patient who expressed mRNA LMP1EBV group and all NPC WHO-3 patients (Table 1.) The positivity of mRNA LMP1 EBV from FFPE NPC WHO-3 tumour biopsy that were 14 of 27 samples (51.9%). The results of this research indicated that the RT-PCR technique could be used to detect the expression of EBV latent gene in NPC WHO-3patients. In this study, the positivity of mRNA LMP1 EBV expression from FFPE tumour biopsy in NPC WHO-3 patients (51.2%) was higher than the previous study in LMP1 expression analysis by Immunohistochemistry technique (10 - 50%) (Middeldorp et al, 2003). However, it was lower than that of mRNA LMP1 EBV expression from the fresh biopsy in NPC WHO-3 patients (91.3%) (Wahyono et al., 2016), that of LMP1 protein expression in NPC by Immunohistochemistry (IHC) staining methods (65%) (Niedobitek, 2000). LMP1 protein expression was detected 75% cases with Immunohistochemistry staining methods and has a significant relationship to locoregional progressivity at the young age (Adham et al., 2012).

The analysis of formalin-fixed paraffin-embedded (FFPE) often has difficulty because of total RNA yielded from FFPE tissue often significantly degraded. Only 3% total RNA is able to isolate from paraffin blocks to obtain cDNA synthesis, compared with the use of fresh biopsy or fresh frozen biopsy (Gouveia et al., 2014). Therefore, fresh biopsy or fresh frozen biopsy more often to be used to analyse mRNA expression than formaline-fixed paraffin-embedded (FFPE) biopsy. However, formalin-fixed paraffin-embedded (FFPE) biopsies have been collected as an archived specimen abundantly at Departement of Pathology Anatomy, Prof. dr. Margono Soekarjo Government Hospital, Purwokerto. Detection of mRNA LMP1 EBV expression from FFPE tumour biopsy in NPC WHO-3 patients by RT-PCR technique is the novelty of the research because of the first research used FFPE tumour biopsy specimens in the analysis of mRNA EBV in NPC.

Table 1. Molecular characteristic and clinical pathologyof research sample

Parameter	Number of Samples
Sample size	27
mRNA LMP1 EBV expression of NPC WHO-3 patient	
Positive	14
Negative	13
Positivity of mRNA EBV of NPC WHO-3 LMP1	51.9%

Identification of the substance that gives information of malignant tumours such as EBV gene expression is expected to improve diagnosis, determine prognosis, and predict NPC pathology (Kresno, 2011). More than 90 percent of the world's population has been infected by EBV that began in the first year after birth (Thompson & Kurszrock, 2004). NPC is a malignancy associated with EBV having epithelial cells as target cell (Young & Rickinson, 2004). LMP1 is more potential than that of VCA-IgA serology and detection of LMP-1 EBV with PCR technique in the nasopharyngeal swab. It can be used as a good diagnosis of NPC pathogenesis (Hao et al., 2004; Cho, 2007). At the time, some of mRNA EBV gene has been used to be a molecular marker in pathogenesis of NPC, such as Epstein-Barr virus Encoded RNA (EBER), Epstein-Barr

virus Nuclear Antigen (EBNA1), Latent Membrane Protein 1 (LMP1), Latent Membrane Protein 2A/2B (LMP2A/2B), BamHI A Fragment Rightward Reading Frame (BART), BamH1 A Fragment Rightward Reading Frame 1 (BARF1), and BamH1 Z Fragment Leftward Reading Frame 1 (BZLF1). However, study exploring the expression of mRNA EBV gene from FFPE tumour biopsy in NPC WHO-3 patients in the pathogenesis of NPC is rarely done (Hirankarn et al., 2004).

Several molecular techniques have been used to detect EBV genes in KNF before, such as in situ hybridization of EBV DNA (1991-1992), hybridization of EBV DNA Blots (1992), PCR DNA EBV (1993-1994), in situ hybridization EBERs (1995 – at present), immuno histochemistry LMP1 (1995), immuno histochemical EA (early antigen difuse) and gp350/220 (2002) (Barnes et al., 2005). RT-PCR technique is the development of Northern blotting techniques that have been commonly used to detect mRNA EBV. The advantage of the RT-PCR technique is that it is more sensitive to detect mRNA and requires a small sample (Brink et al., 1997; Middeldorp et al., 2003). The multiprime RT-PCR technique is a variation of conventional RT-PCR techniques to simultaneously detect mRNA expression of several EBV genes EBNA1, EBNA2, LMP1, LMP2A, LMP2B, BZLF1, BARTs, and U1A snRNP (house keeping gene) (Steven et al., 2005). Therefore, RT-PCR-based molecular technique is the preferred technique for the analysis of mRNA LMP1 EBV expression on NPC pathogenesis.

LMP1 EBV as the parameter of NPC diagnosis is more potential than serological detection by VCA-IgA. The detection of LMP-1EBV by PCR technique on Nasopharyngeal swab can be used as a good diagnostic for NPC pathology (Cho, 2007; Hao et al., 2004). Constitutive activation of NF-kB by the viral oncogene (LMP1) has an important role in persistence, but is a risk factor for EBV-associated lymphomas. Endogenous LMP1 escapes degradation upon accumulation within intraluminal vesicles of multivesicular endosomes and secretion via exosomes. LMP1 associates and traffics with the intracellular tetraspanin CD63 into vesicles that lack MHC II and sustain low cholesterol levels, even in 'cholesterol-trapping' conditions. The lipid-raft anchoring sequence FWLY, nor ubiquitylation of the N-terminus, controls LMP1 sorting into exosomes. Rather, C-terminal modifications that retain LMP1 in Golgi compartments preclude assembly within CD63-enriched domains and/or exosomal discharge leading to NF-kB overstimulation. Interference through shRNAs further proved the antagonizing role of CD63 in LMP1-mediated signalling. Thus, LMP1 exploits CD63-enriched microdomains to restrain downstream NFjB activation by promoting trafficking in the endosomalexosomal pathway. CD63 is thus a critical mediator of LMP1 function in- and outside-infected (tumour) cells (Verweij et al., 2011). mRNA LMP1 EBV that is a transcript of BNLF1 gene are detected abundantly in cell culture (Boos et al., 1987; Middeldorp et al., 2003). mRNA LMP1 EBV is used to be a marker of EBV infection in NPC (Yu & Yuan, 2012), because LMP1 EBV plays an important role for transformation from normal cells into tumour cells and metastatic tumour cells (Barnes et al., 2005; Cho, 2007). Therefore, the expression of LMP1 mRNA can be used to perform NPC screening and as a marker of NPC diagnostic molecules. The positivity of mRNA LMP1 EBV expression is 10-50 percent (Middeldorp et al., 2003). In previous studies, free EBV DNA in plasma were detected in 3 of 17 healthy people as control sample (18%) and the expression of EBV lytic genes could be detected in healthy career tissue biopsies (Martel-Renoir et al, 1995; Feng et al., 2000; Gulley, 2001).

FFPE is routinely used for the histopathological diagnosis like cancer in the hospital. The process of fixation and paraffin block embedded is able to change the structure of DNA as formaline fixation process. Fixation process of the network tissue using the formalin and stored it for long periods can cause changes in the structure of cells and also causing DNA degradation. Fixation should be used for optimum results is the use of liquid neutral buffered formalin, compared with 10% of non-buffered formalin as it will slow down the process of degradation caused by formaldehyde (Rezeki et al., 2014). The advantages of using FFPE NPC tumour biopsy in this researchs i.e. FFPE NPC tumour biopsy have a potensial tissue bank which it has been collected by Pathology Anatomy Department of Prof. dr. Margono Soekarjo Hospital for many years, FFPE NPC tumour biopsy was a non-invasive clinical sample because it was an archived specimen, FFPE NPC tumour biopsy needed not an ethical approval, the molecular histopathological diagnosis used by FFPE NPC tumour biopsy was rarely used until at present. Therefore, the expression of mRNA LMP1 EBVFFPE tumour biopsy in NPC WHO-3 patients can be used to be an NPC diagnosis.

The research result indicated that mRNA LMP1 EBV from FFPE tumour biopsy in NPC WHO-3 patients were expressed moderately (51,2

%). The expression of mRNA LMP1 EBV from FFPE NPC tumour biopsy has potential as a tumour biomarker of NPC diagnosis in that NPC management. Therefore, the molecular methods of NPC diagnosis would improved the management of NPC.

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