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RESEARCH ARTICLE

Latent Membrane Protein 1 Gene of Epstein - Barr virus: 30-Base-Pair Deletion in Indonesian Nasopharyngeal Carcinoma Patients

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Abstract

Introduction: Epstein Barr Virus (EBV), a member of the Herpesviridae family, is a dsDNA virus. Nasopharyngeal carcinoma **(**NPC) patients express EBV latent genes EBERs, EBNA1, LMP1, LMP2A, and LMP2B. The LMP1 gene, in particular, plays important roles in epithelial oncogenesis and B-lymphocyte transformation. Several epidemiological studies found a specific variant of LMP gene detectable as a 30-bpdeletion in NPC patients and it is still unclear whether this deletion is associated with NPC pathogenesis in Indonesian population. The aim of this research is to understand the existence of the deletion of the 30-bp LMP1 gene in Indonesian NPC patients and to determine the frequency of 30-bp deletion of LMP1 gene and its association with the pathological status. Methods: The 30-bp deletion in LMP1 gene was identified by nested PCR and subsequent electrophoresis in 2% agarose gel. The results were determined as 162-bp DNA band of the LMP1 gene (without 30-bp deletion) and 132-bp DNA band of the LMP1 gene (with 30-bp deletion). Results: Among 100 identified samples from Jakarta, 8 samples were found to have the 30-bp deletion, 71 samples did not have 30-bp deletion and 21 samples carried coexistence variants. Conclusion: In Indonesia, especially in Jakarta, EBV variant of 30-bp deletion of LMP1 gene was found at low frequency (8%) in comparison with variant without deletion (71%). There is a variant of LMP1 gene mixtures (21% coexistence with and without deletion). Analysis of data using Fisher's exact test shows that there is no significant relationship between 30-bp deletion of LMP1 gene and NPC pathological status ($p > 0.05$).

Keywords: *30-bp deletion, NPC, EBV, LMP1, Coexistence.*

Introduction

The Epstein-Barr virus (EBV) is a dsDNA virus, has an icosahedral capsid, and belongs to the Herpesviridae family. EBV infection can be associated with several diseases such as Burkitt's lymphoma, T-cell lymphoma, infectious mononucleosis, and nasopharyngeal carcinoma (NPC). [1]. NPC is a malignant tumor that occurs in epithelial cells in the nasopharynx region, in the Rosenmuelleri basin and eustachian duct. Many factors are suspected to be associated with NPC, namely: 1. the presence of EBV infection, 2. Environmental factors including living habits, 3. Genetic susceptibility (mongoloid ethnics) [2]. From several studies, it was found that NPC is consistent with EBV infection.

The studies of Shotelersuk *et al* [3]. Showed that EBV DNA in plasma can be used to establish NPC diagnosis. In NPC patients, latent EBV genes EBERs, EBNA1, LMP1, LMP2A, and LMP2B are expressed. EBNA1 protein plays a role in maintaining viral DNA in latent infection. The transmembrane proteins LMP2A and LMP2B inhibit the tyrosine kinase signal, which is believed to inhibit viral lytic cycles. LMP1 protein plays a role for EBV-related cell transformation and of all latent EBV genes, LMP1 gene is most important in cell transformation [4]. The LMP1 protein structure comprises 386 amino acids, divided into 20 amino acids at the terminal N, 6 transmembrane protein segments (166 amino acids) and 200 amino

acids at the terminal carboxy (C) [4]. The transmembrane protein LMP1 mediates tumor necrosis factor (TNF) signals and increased regulation of IL-10 cytokines that proliferate B-cells and inhibit local immune responses [4]. Several epidemiological studies have reported high prevalence of NPC caused by EBV infection in Asia. In southern China precisely in Guangdong province, NPC cases occupy the highest place, 2,500 new cases per year with a prevalence of 39.84/100,000 inhabitants, while prevalence in southeast Asia is reported to be 3/100,000 in the indigenous Thai population and 10/100,000 in the population of Thailand that has been assimilated to ethnic Chinese [5].

In Malaysia, the NPC incident ranks second-most. High frequency is found in Malaysian population of ethnic Chinese descent. The incidence is 18.1/100,000 in males and 7.4/100,000 in females [6]. These figures are much higher compared to Europe or North America with a prevalence of 1 per 100,000 populations per year [1, 7-8].

Based on epidemiological studies of NPC and its association with EBV infection, a special variant was widely found with a 30-bp deletion in the C-section of the LMP1 gene terminal (delta 30-bp LMP1 gene). The 30-bp LMP1 gene deletion was found in the Chinese population of NPC patients, as reported by Hu *et al.* [9], Khanim *et al.* [10] and Lin *et al.* [11]. This deletion was also found in European population of NPC [10], but not in Russian NPC patients [12]. To date it is not known whether the delta 30-bp LMP1 gene affects EBV pathogens in NPC.

In Indonesia, the most malignant head and neck cancers are NPC cases ranking fourth after carcinoma of the cervix, mammary and skin. The incidence rate is about 4.7 new cases per 100,000 population per year and is 2-3 times more common in men than in women [8]. An Indonesian study in Malang (East Java) concluded that 98% of NPC patients in Indonesia NPC are associated with EBV infection [13]. From the results of various studies, to date there has been no complete research publication of the genetic variants of EBV, especially the 30-bp deletion of the LMP1 gene that may be associated with pathogenesis and malignancies of NPC in Indonesia. The aim of this study was to find out if there was a deletion of 30-bp of LMP1 gene in NPC patients from Jakarta, knowing the deletion frequency of 30-bp LMP1 gene in NPC patients and 30-bp deletion in LMP1 gene with pathology status of NPC in Indonesia.

Methods

Research Subjects

Research subjects were NPC patients who had been shown to be exposed to EBV and were undergoing therapy. All subjects of the study had previously provided informed consent before being included in the study. The diagnosis of NPC was established based on histopathology examination of biopsy tissue conducted in the Department of Ear, Nose, Throat, Head and Neck (ENT-HN) FMUI / RSCM Jakarta.

Furthermore, the NPC stage was defined by the classification made by the American Joint Committee on Cancer (AJCC) and the Union International Controle Cancer (UICC). The primary NPC patient in the study classified the tumor by a pathologist in the Department of Anatomical Pathology FMUI / RSCM based on the WHO criteria that included patients with type I, II, and III tumors. Demographic data and clinical information were obtained through interviews with patients using patient questionnaires and medical records.

Sample Determination and Criteria

The study samples were 3 mL of peripheral blood from NPC patients without age and sex restriction. Sampling of NPC patients was done in the Department of ENT-HN and Radiotherapy RSCM Jakarta, while the identification of genes was conducted in the Laboratory of the Department of Medical Biology FMUI. The number of samples was calculated according to the number of prevalence study samples or single proportion with the following formula [14-15]:

$$n = \frac{Z_{\alpha^2} pq}{d^2} = \frac{4pq}{d^2}$$

With: α = 5% (level of significance 95%); p = 0.5 (estimated proportion of 30-bp LMP1 gene deletions); q = 1 – p; d = + 15% = 0.15 (accuracy of p-value).

From this calculation the value obtained for n was 42.68 and fitted to 50 samples.

Isolation of EBV DNA from Serum [16]

The peripheral blood samples (3 mL) from NPC patients were centrifuged in a vacutainer to separate the blood into serum and blood cells. Subsequently, EBV DNA was isolated: serum (100 µL) were placed into a 1.5 mL Eppendorf tube, added 300 µL Tris-EDTA buffer (TE), 50 µL sodium dodecyl sulfate lysis buffer **(**SDS) 10%, and 1 µL proteinase-K. The mixture was vortexed for 1 minute (min) and incubated at 65^{0}C for 1 hour (h). After incubation was completed, 200 µL of phenol and 200 µL chloroform/iso-amyl alcohol (C-IAA, 24:1) were added.

The mixture was vortexed for 3 min and centrifuged at 10,000 rpm for 10 min at 20^{0}C. The latter was moved into a new Eppendorf tube, 100 µL of phenol and 300 µL C-IAA were added, the mixture again vortexed for 3 min and centrifuged at 10,000 rpm for 10 min at 20^{0}C. The above procedure was repeated 2-3 times until a clear aqueous layer was obtained, which was transferred into a new Eppendorf tube. Subsequently, 30-40 µL of sodium acetate, pH 4.2 (1/10 volume) and 1 mL of cold absolute ethanol were added to precipitate DNA by flipping the Eppendorf tube several times. The tube was stored overnight at -20^{0}C. The next day, the Eppendorf tube was centrifuged at 12,000 rpm and 20^{0}C for 30 min until a white pellet was visible at the bottom of the tube. The supernatant was discarded, and 70% cold sterile ethanol added to wash the DNA by centrifugation at 10,000 rpm for 10 min at 20^{0}C. The supernatant was carefully removed, and the DNA dried for 30 min by tilting the Eppendorf tube on tissue paper. The dried DNA pellets were then rehydrated with 20 µL TE and stored at -20^{0}C for further analysis.

Amplification of LMP1 Gene DNA

Amplification of the LMP1 gene was performed using repeated PCR (nested-PCR). For the first PCR step, the outer primers used refer to Khanim *et al*. [10] (forward 5'-GACATGGTAATGCCTAGAAG-3'and reverse 5'-GCGACTCTGCTGGAAATGAT-3'). For the second PCR step, inner primers were designed using Primer3 online software [17-18] (forward 5'-GTCATCATCTCCACCGGAAC-3' and reverse 5'-CCACAATTGACGGAAGAGGTT-3').
Before starting DNA amplification by nested PCR, optimization towards the specific primers used was accomplished to determine the most appropriate annealing temperature. The optimized PCR conditions used for the NPC samples can be seen in Table 1.

Table 1: The conditions of nested PCR of the LMP1 gene

Nested PCR	PCR Condition PCR Product Denaturation Annealing Extention (bp)
First PCR (Outer primer) 5'-ACATGGTAATGCCTAGAAG-'3 5'-GCGACTCTGCTGGAAATGAT-3'	94^{0}C, 30" 55^{0}C, 90" 70^{0}C, 120" 260
Second PCR (Inner primer) 5'-GTCATCATCTCCACCGGAAC -3' 5'-CCACAATTGACGGAAGAGGTT-3'	94^{0}C, 30" 59^{0}C, 90" 70^{0}C, 120" 162

The volume of each PCR reagent was 25 µL, consisting of 10 µL EBV DNA, 2.5 µL of 10x $MgCl_2$-free buffer, 2.5 µL $MgCl_2$ (25 mM), 0.5 µL dNTP (10 mM), 0.5 µL forward primer, 0.5 µL reverse primer, 0.125 µL Taq DNA polymerase, and 8.375 µL ddH_2O. The PCR reagent solution was mixed at low temperature in an Eppendorf tube, centrifuged for several seconds (sec) to homogenize the mixture and minimize the amounts of reagents attached to the wall of the Eppendorf tube.

Then, one drop of mineral oil was added to the PCR reagent solution to avoid evaporation in the PCR machine. The PCR reagent solution was loaded into the programmed PCR machine for the first PCR step. The denaturation stage was carried out at 94^{0}C for 30 sec, the primer annealing stage at 55°C for 90 sec, and extension at 70^{0}C for 120 sec. These three stages were repeated up to 30 cycles. The first PCR product was used as the DNA template for the second PCR.

The volume of each PCR reagent was the same as in the first PCR step, the only difference were the primers used. The PCR machine was programmed according to optimal conditions for the second PCR: denaturation was carried out at 94°C for 30 sec, primer annealing at 59°C for 90 sec, and extension at 70°C for 120 sec; the three stages also repeated up to 30 cycles.

Detection of LMP1 Gene Amplification Results

Detection of PCR results was performed by electrophoresis with 2% agarose gel at 90 Volts for 55 min.

The non-deleted LMP1 gene presented as a 162-bp band, while the deleted LMP1 gene did as a 132-bp band. After electrophoresis and emerging bands of the size corresponding to the primer, DNA sequencing was performed. The ordered samples were representative of each of the 162-bp and 132-bp DNA bands when detected by electrophoresis. The sequenced samples were then used as a marker for each following electrophoresis process.

LMP1 Gene Analysis

To analyze the relationship between 30-bp deletion and NPC pathology status, Chi-square statistic test was used. If the data obtained did not qualify for Chi-square test, the statistical procedure was continued by Kolmogorov-Smirnov test. The result was considered significant at $p < 0.05$ [14].

Results

Using nested-PCR, all samples of collected NPC patients could be identified. Electrophoresis results can be seen in Figure 1, while the overall research results can be seen in Table 2.

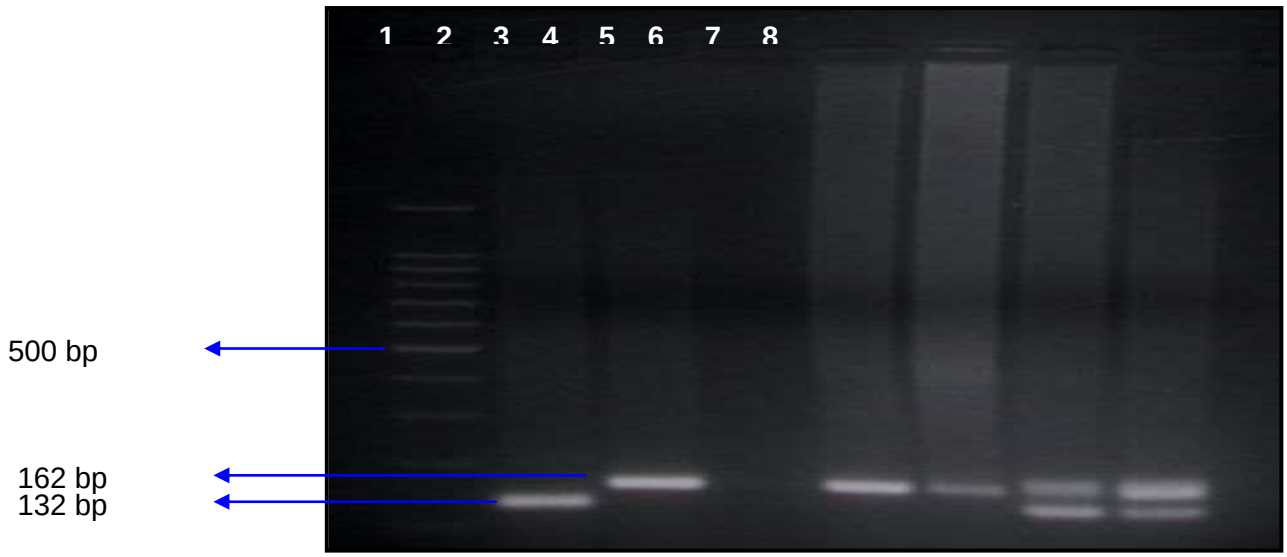


Figure 1: Agarose gel electrophoresis of nested PCR results

Lane 1: DNA ladder from 100-bp; lane 2: Sample number 34 as control of 132-bp; lane 3: Sample number 25 as control of 162-bp; lane 4: Negative control; lanes 5-6: Samples without deletion = 162-bp; lanes 7-8: Samples with two bands (132 and 162-bp)

Table 2: Results of nested PCR in NPC patients

Identification of LMP1 gene bands	Number of Patients	Percentage (%)
132-bp	8	8
162-bp	71	71
132 and 162-bp	21	21
Total	100	100

Out of 100 samples from NPC patients, there were 8 samples showing a specific band at 132-bp. The 162-bp band was detected in 71 samples that did not have deletion at all and 21 samples with both bands in question (132 and 162-bp). Thus, only 8% of the samples had a 30-bp deletion in the LMP1 gene, whereas the specific band of 162-bp was existent in 71% of the samples and 21% carried coexistence with and without deletion, 132 and 162-bp

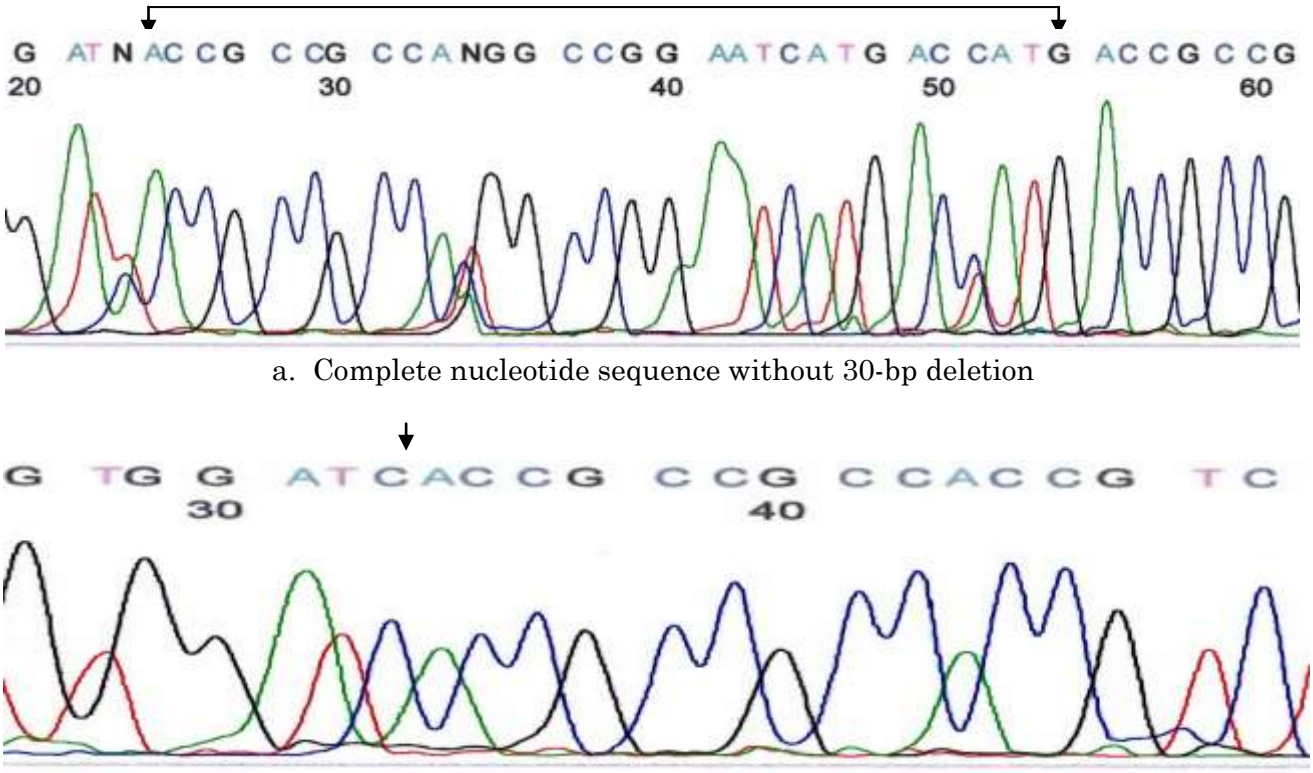
If the nested PCR results are associated with early and late stages of NPC patients, the results can be seen in Table 3.

Table 3: Results of nested PCR associated with early and late stages of NPC patients

Identification of LMP1 gene bands	Early stage		Late stage	
	Amount	Percentage (%)	Amount	Percentage (%)
132 bp	1	7.7	5	6.7
162 bp	9	69.3	55	73.3
132 and 162 bp	3	23.0	15	20.0
Total	13	100	75	100

Note: Amount = number of patients; there was no significant difference ($p > 0.05$) between NPC patients at early and late stage

Sequencing results of samples 25 and 34 used as controls are shown in Figure 2. The deleted region is present in the nucleotide sequence of sample 25 denoted from 24th until 54th nucleotides, whereas in sample number 34, the deleted nucleotide sequence between the 33rd and 34th nucleotides is not present.



a. Complete nucleotide sequence without 30-bp deletion

b. Nucleotide sequence with a 30-bp deletion

Figure 2: Results of DNA sequencing. (A). DNA sequencing result of sample number 25 with an area of LMP1 gene that did not have a 30-bp deletion. Deletion occurs in the marked region between the 24th and the 54th nucleotides. (b). DNA sequencing result of sample number 34 with an area of the LMP1 gene having a 30-bp deletion between the 33rd and 34th nucleotides marked by an arrow Based on the data of all samples, there were 12 samples with incomplete data on the pathological status; hence, only 88 samples were statistically tested for relation to pathology. Chi-square test performed was not fulfilled and the statistical procedure continued with Kolmogorov-Smirnov test. The result obtained was a p-value of 0.996 meaning that there was no significant relation between the 30-bp deletion in LMP1 gene and the NPC pathological status (p>0.05)

Discussion

Nasopharyngeal carcinoma is a multifactorial disease. One of the factors causing NPC is EBV infection [2]. This can be ascertained because in serum or plasma of NPC patients EBV DNA can always be found [3, 19]. Epstein-Barr virus will express several latent genes, such as EBERs, EBNA1, LMP1, LMP2A, and LMP2B [4]. Among these latent genes, the LMP1 gene plays an important role in the transformation of B-lymphocytes by EBV. In addition, LMP1 is also believed to play a role in the oncogenesis. The LMP1 protein can protect the infected cells from apoptosis and increase cell proliferation [20, 21].

Based on the epidemiological studies of NPC, especially regarding the LMP1 gene, there is one distinctive variant of the LMP1 gene, the 30 bp deletion in the C-terminal section. The C-terminal region is thought to affect oncogenesis [6]. Deletion of 30-bp of the LMP1 gene is commonly seen in diseases associated with EBV infection, such as Hodgkin's lymphoma, Burkitt's lymphoma, and infectious mononucleosis [10]. In this study, a deletion of 30 bp LMP1 gene could be detected using nested PCR. In the first PCR step using outer primers, not all reactions showed specific bands; only 4 out of 43 samples yielded a specific band of 260-bp.

This may be due to the low concentration of viral DNA, which may not be visible in electrophoresis. But in the second PCR step using inner primers, all samples exhibited a specific DNA band. This study showed 30-pb deletion in the LMP1 gene of 24% of NPC sufferers. This result is certainly much lower than in other Asian countries where the NPC

incidence is included in the highest order. The low rate of 30-bp deletion in this study cannot be fully explained.

However, the results of this study are not much different from the research conducted by See *et al.* [7] in Malaysia, where 24.1% (7 of 29 samples) of blood plasma samples from NPC patients exerted 30-bp deletion. Regarding the incidence rate of NPC, Malaysia is a country with a high prevalence. According to the results of Devi *et al.* [22] the NPC cases in Malaysia-Sarawak have a prevalence of 13.5 per 100.000 populations. According to some studies, the deletion of 30-bp of the LMP1 gene is identical with the NPC cases in Asia [23].

In endemic areas of NPC, namely South China and Taiwan, the 30-bp deletion rate can reach 70% or even up to more than 90% [7]. Deletion of 30-bp of the LMP1 gene occurs in the C-terminal section and lies between amino acids 343 and 352 [24]. Within the LMP1 protein structure, the deletion lies in the TES2 / CTAR2 section. CTAR2 can bind to TRADD / TRAF2 and mediates 66% of NF-κB activity.

The NFκB activity through LMP1 will regulate the expression of several other genes associated with biological processes and the role of LMP1 in tumorigenesis of NPC [25]. In NPC patients, LMP1 initiates cascade signals through a bonding complex between TRADD / TRAF2 and CTAR2. LMP1 can manage the expression of cyclin D1. LMP1 increases the regulation of cyclin D1 transcription through the NF-κB signal pathway; this may cause the cell cycle shift from G1 to S phase to occur earlier.

Increased regulatory transcription of cyclin D1 may lead to decreased G1 / S checkpoint regulation. This will result in abnormal cell proliferation [25]. According to a study conducted by Fries *et al.* [26], the expression of LMP1 gene on T-cells, B-cells, and epithelial cells can protect them from p53 apoptosis pathway by increasing Bcl2 and A20 regulation as anti-apoptotic genes. In epithelial cells infected with EBV, LMP1 will induce A20 to protect epithelial cells from p53 apoptosis pathway [26]. In addition to the increase in Bcl2 and A20, apoptosis in NPC patients is also inhibited by survivin expression. Survivin is an apoptosis inhibitor protein. LMP1 can stimulate survivin expression through NF-κB. In the cell cycle, survivin is generally expressed during the G2 / M phase. In epithelial cells of NPC patients, survivin is not only found in G2 / M phase, but can also be seen in the phases G1 and S.

The survivin expression in G1 / S phase is modulated by LMP1. Hence, LMP1 contributes to inhibiting apoptosis of NPC cells [25]. According to some studies, the presence of a 30-bp deletion of LMP1 gene will play a role in improving tumor progression. Two LMP1 strains having a 30-bp deletion i.e., CAO and C1510 can increase tumorigenesis when compared to strain B95.8, which is the EBV wildtype strain [27]. Research conducted by Knecht *et al.* [28] shows that the 30-bp deletion is associated with malignant disease rates.

The association is also shown from other studies: in 30% of Hodgkin's lymphoma patients and 65% of peripheral T-cell lymphoma (PTL) patients in Europe, as well as in 100% of PTL patients in Malaysia [29]. Research conducted by Santón *et al* [30]. In Spain showed histologically and clinically an increase in tumor aggressiveness in Hodgkin's lymphoma patients who had a deletion of 30-bp in the LMP1 gene. Fisher's exact test results in a p-value of 0.745 in this study.

Thus, the hypothesis is not accepted (since $p>0.05$) indicating that there is no significant relationship between 30-bp deletion in LMP1 gene and the pathological NPC status. The results of this study are indeed different from some previous research which stated the 30-bp deletion in LMP1 gene being associated with tumor malignancy rate. In this study, 30-pb deletions were found in sample number 21, 24, 34, 72, 74, 82, 96, and 97.

The staging data of the respective samples differed, one sample was at stage II, 2 samples were at stage III, 3 samples at stage IV, and 2 samples had no pathology status. Kolmogorov-Smirnov test showed no significant relationship between 30-bp deletion inLMP1 gene and NPC pathology status. Research conducted by Dolcetti *et al.* [23] also showed the same result: the 30-bp deletion was not associated with EBV risk factors for the development of Hodgkin's disease. According to Hahn *et al.*, [12] there is no relationship between the 30-bp deletion and the induced level of NF-κB, so that the 30-bp deletion is not a predisposing factor for NPC cases in Russia [12].

In Taiwan, the 30-bp deletion is the dominant viral variant in NPC patients, present in 82.3% of NPC patients [31]. Nevertheless, in a retrospective cohort study, 30-bp deletion was statistically unrelated to NPC prognosis. The 30-bp deletion in the LMP1 gene is a polymorphism only in the endemic areas of NPC [31]. The results of this study resemble those from Taiwan: the 30-bp deletion in Indonesia is a polymorphism phenomenon in the LMP1 gene. In epidemiological studies of NPC, the incidence of a 30-bp deletion in the LMP1 gene differs in each population due to the geographical location.

In general, Asia as an NPC endemic region has high rates of deletion compared to other regions. According to the data obtained, NPC is the most malignant ENT tumor in Indonesia and the 4th after cervical, breast, and skin cancers [8]. Another Indonesian study in Malang (East Java) using biopsies found a 30-bp deletion with 151-bp and 18-bp polymorphism in the EBV B95-8 region from 168209 to 168320. The authors conclude that 98% of NPC patients in Indonesia NPC is associated with EBV infection [13].

Nevertheless, the results of this study do not support this statement: in Jakarta, the 30-bp deletion in NPC patients is very low at only 8%. Such low 30-bp deletion rates are generally obtained from non-endemic NPC areas. Interestingly, this study found two LMP1 gene variants (132 and 162-bp) in 21% of the blood samples from NPC patients. This is possible if more than one infection occurred by EBV with different LMP1 gene variants.

Similarly, previous research conducted by Tan *et al*. [32] found 2 variants of the LMP1 gene (132 and 162-bp) in biopsy samples from NPC patients. The study of See *et al*. [7] also found the coexistence of two LMP1 gene variants using samples from biopsies and blood plasma of NPC patients. Five blood plasma samples out of 29 NPC patients (17.2%) had coexistence of 2 LMP1 gene variants, whereas in biopsy samples no coexistence of 2 variants was found. According to the authors, the results of their research were the first cases of the coexistence of 2 LMP1 gene variants obtained from blood plasma, but not in biopsy. In general, the EBV variant in blood plasma is a feature of the EBV variants from NPC tumor biopsies [7].

Re-reading the sequence of sample number 25, the 30-bp deletion occurs in the following order: 5'-accgccgccaNgcggggaatcatgaccatg-3'. This corresponds to the LMP1 nucleotide sequence data in the Gene Bank and the 30-bp deleted DNA sequencing data performed by Higa *et al*. [33] using the B95.8 strain. In the sequencing result the 11th nucleotide is unreadable.

Based on data from Gene Bank and Higa *et al*. [33] the unreadable nucleotide in the 11th sequence is t. According to Miller, the deletion does not affect the process of reading the amino acids 342-352: Gly-Gly-His-Ser-His-Asp-Ser-Gly-His [34].

Conclusion

There was a deletion of 30-bp of LMP1 gene in NPC patients in Indonesia especially in Jakarta. All the 100 samples of NPC patients, 8% of the samples were deleted and 71% of the samples were not deleted. In this study found coexistence LMP1 gene variants as much as 21%. There was no significant association between the 30 bp LMP1 gene deletions with NPC pathology status in Indonesia.

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