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Sequence variation of latent membrane protein 2A (LMP2A)gene from Epstein-Barr virus epitope cytotoxic T-lymphocyte (CTL)related to human leucocyte antigen-A24 (HLA-A24)in peripheral blood sample and cytobrushof nasopharyngeal cancer patients

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ABSTRACT

Epstein-Barr virus **(**EBV) infects lymphocyte B and triggers latent phase in the host so that it causes nasopharyngeal carcinoma (NPC). Latent membraneprotein 2A (LMP2A) epitope CTL-HLA-A24 is a target for recognition by cytotoxic T lymphocyte(CTL). The change in the epitope could influence the latency of particular EBV in the host due toits ability to evade immune surveillance mediated by CTL. The study aimed to determine thesequence variation of LMP2A epitope CTL-HLA-A24 gene from the peripheral blood samples and cytobrush of the NPC patients. Case-series study was conducted with total 16 cytobrush samples from NPC patients. DNA isolation, polymerasechain reaction (PCR) and gene sequencing were performed in this study. From cytobrush samples of NPC patients, it was found the changes of base sequence variation of LMP2A gene from GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC and TCT>ACT. CCA>CCC and TGC>TCC variations were found in epitope associated with HLA-A2 where there was a change of epitope sequence from TYGPVFMCL to TYGPVFMSL caused by missense mutation. The change of base sequence caused amino acid alteration from cysteine to serine. Whereas the variation of CCA>CCC did not change the sequence of amino acid proline so that the epitopewas unaffected. In epitope associated HLA-A2 (CLGGLLTMV), there was a change in base sequence from GGT to GGC, but there was no changes in amino acid and still as glycine. There were some new variations: in the upstream sequence of LMP2A from GGC>GGA which is silent mutation and the other variation is in downstream sequence of LMP2A from TCT>ACT which is missense mutation. Thesequence variations of LMP2A gene found in this research were GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC and TCT>ACT. In our research, we found another variation compared the previous research. The variation was in the upstream sequence of LMP2A from GGC>GGA which is silent mutation and the other variation is in the downstream sequence of LMP2A from TCT>ACT which is missense mutation.

ABSTRAK

Epstein Barr Virus **(**EBV) menginfeksi limfosit B dan laten didalam tubuh *host* sehingga menyebabkan karsinoma nasofaring (KNF). Gen*latent membrane protein 2a*(*Lmp2a*) epitop CTL-HLA-A24 merupakan target *cytotoxic T lymphocytes* (CTL). Perubahan epitop tersebut dapat mempengaruhi latensi EBV dalam inang karena menghindari system imun yang dimediasi oleh CTL. Penelitian ini bertujuan untuk menentukan variasi sekuen gen LMP2A epitop CTL-HLA-A24 dari sampel darah tepi dan *cytobrush* penderita KNF. Rancangan penelitian *Case-series* digunakan pada penelitian ini. Sebanyak 16 sampel *cytobrush* penderita KNF dilakukan isolasi DNA, pemeriksaan *polymerascechain reaction* (PCR) yang dilanjutkan dengan sekuensing. Pada hasil sekuen sampel *cytobrush* penderita

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KNF, ditemukan adanya perubahan variasi sekuen gen LMP2A yaitu perubahan susunan basa dari GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC dan TCT >ACT. Variasi CCA>CCC dan variasi TGC>TCC ditemukan pada epitope terkait HLA-A24 dimana terdapat perubahan epitope TYGPVFMCL menjadi TYGPVFMSL yang disebabkan oleh *missense mutation*, perubahan urutan basa TGC menjadi TCC dimana asam amino yang disandi berubah dari sistein menjadi serin. Sedangkan variasi CCA>CCC tidak mengubah susunan asam amino yang disandi yaitu prolin sehingga tidak merubah epitop. Pada epitop terkait HLA-A2 yaitu CLGGLLTMV, terjadi perubahan urutan basa GGT menjadi GGC, akan tetapi perubahan ini tidak merubah asam amino yang disandi yaitu sama-sama menyandi asam amino glisin. Variasi baru ditemukan pada bagian *upstream* sekuen LMP2A yaitu GGC>GGA yang berupa *silentmutation* dan pada bagian *downstream* sekuen LMP2A yaitu TCT>ACT yang berupa *missense mutation*. Variasi sekuen gen LMP2A yang ditemukan adalah perubahan susunan basa dari GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC dan TCT>ACT. Dalam penelitian ini ditemukan variasi yang berbeda dengan penelitian sebelumnya yaitu pada bagian *upstream* sekuen LMP2A yaitu GGC>GGA yang berupa *silentmutation* dan pada bagian *downstream* sekuen LMP2A yaitu TCT>ACT yang berupa *missense mutation*.

Keywords:
NPC
EBV
Lmp2a
CTL
HLA
A24

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial tumor located on the surface of the nasopharynx. NPC is a malignant tumor that mostlyinvades the head and neck. This carcinoma is rare in some countries in the world, especially in Europe and North America.[1] However, manyare distributed in a particular ethnic and geographic area.[2] The incidence of NPC is high in some areas of southern China, especially in Canton, Guangzhou that is 30-80/100,000 per year.[3] NPC cases in Indonesia continue to increase over time. In Yogyakarta Special Region, the incidence of NPC is increased each year.[4,5]

Epstein-Barr virus (EBV) is proven to be responsible for the occurrence of NPC andcommonly associated withthe progression of the disease.[2,6] EBV is the main virus that caused mononucleosis infection, mainly found in nasopharyngeal tumour cells but not all the lymphocytes.[2] Besides virus, there are other risk factors that could causeNPC such as ethnic, smoking, genetic and gender. EBV infects nearly 95% percent of world population and not only caused cancer disesases.[7]

EBV enters the human body by saliva through oropharynx mucosa and infects B cell residing in the submucosa by CD21-Gp350 bond between them. After that, EBV enters B cell's cytoplasm and dissembles its body and let its genetic material to enter the nucleus. EBV can undergo a latent phase after infecting B cell and reside in the body of the host for all the time.[6] EBV-infected B cells will produce latent antigens such as Latent Membrane Protein (LMP)1 and LMP2 (LMP2A and LMP2B). LMP1 is detected approximately 65 and 35% in NPC patients in mRNA and protein levels, whereas LMP2A is found in more than 95% of NPC samples in mRNA levels and 50% in protein level, respectively.[8]

LMP2A is responsible for the latent phase of EBV-infected B cell by inhibiting or disrupting B cell's signal transduction, keeping the virus in the latent phase/ persistence.[6,8-11] It is immunogenic and acts as themain target of cytotoxic T lymphocytes (CTLs) in NPC. CTL detects the virus by recognizing antigen presented by class I MHC (major histocompatibility complex) in surface of target cell. EBV-infected cells will express latent antigens and showed them in the surface in the form of epitope-class I HLA that may cause T cell response (CTLs). Stevens *et al.*[12] have identified so far seven epitope targets by CTL in LMP2A, whereas Khana *et al.*[9] reported that CTL recognizes recombinant LMP2A epitope associated with HLA-A2 in tumour cells having

disturbance in protein TAP regulation, so that this virus isolate evading immune response through the change in amino acid chain in CTL's epitope.[13]

Epitope part of LMP2A associated with CTL-HLA A24 gene has TYGPVFMCL amino acids arrangement. Previous study found that a change in that region in NPC patients could affect the recognition site of the epitope so that CTL could not recognize the new epitope, thus allowing EBV to avoid the immune surveillance of CTLs.[9] HLA–A24, HLA-A11 dan HLA-A2 type are found in NPC patients resides in south east Asia and caucasian.[14]Prior study found that out of nine people (healthy or infected with EBV), HLA-A24 showed the highest number, followed by HLA-A11 and HLA-A2. Native Indonesians with HLA-A24 have high probabilities of NPC.[15]

NPC cases in Indonesia continue to increase due to most patients diagnosed at the late stage, so treatment failureoccasionally occurs. To minimize the number of cases and treatement failure, early screening needs to be undertaken. Never theless, the study of genetic profile in patients with NPC remainsscarce. Therefore, we aimedto find LMP2A-CTL epitope-HLA A24 gene in the peripheral blood and cytobrush of NPC patients, and also peripheral blood of healthy people. In addition, the sequence variation of LMP2A-CTL epitope-HLA A24 gene from those samples will be determined.

MATERIALS AND METHODS

Design and samples

This is a Case Series design towards the occurrence of sequence variation of *Lmp2a*-CTL epitope-*HLA A24* gene from the samples of cytobrush NPC patients. Peripheral blood and cytobrushwere obtained from the NPC patients at ear-nose-throat (ENT) polyclinic in Dr. Sardjito General Hospital, Yogyakarta, Indonesia.

Procedure

DNA isolation and PCR were performedat Laboratory of Molecular Biology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta and sequencing was done at Genetika Science Indonesia – Laboratorium 1st Base.

Method used in DNA isolation is Booms method using kit. NucliSENS® Lysis Buffer, NucliSENS® automated isolation reagent (wash buffer, eluent buffer, silica), aseton, etanol 70%, H_2O steril, HPLC water, platinum taq DNA polymerase (include buffer, $MgCl_2$), 10 mM dNTP mix, Ultra Pure DNAse/RNAse, trisma base, 100bp DNA ladder (marker), lyophilized primer 100 nmol gen lmp2a, ddNTP set Bio Chemika, GF-1 PCR Clean Up Kit, 100bp DNA Ladder, loading buffer, agarose gel electrophoresis, TAE buffer, ethidium bromide.

PCR was performed with 1 µL DNA in a 24 µLtotal reaction mixture containing primer 10pmol 1µL for each forward and reverse, buffer 10x 2.5µL, $MgCl_2$ 50nm 1.5µL, dNTP mix 10nm 0.2µL, Taq DNA Polymerase 0.1 µL.The primer used for LMP2A was5'-CAT TCT TGC TAT CCT GAC CG 3'(forward primer) and5'-CTC CTC ACT TTC CAG TGT AAG G-3'(reverse primer).[9] The amplification protocol was one cycle at 95°C for 5min, followed by 35 cycles of PCR with denaturation temperature 94°C for 1min, annealing temperature 58°C for 50 sec, extension temperature 72°C for one min, final extension 72°C for 5 min, 4°C. Result product has 324 bp. PCR product then under wentelectrophoresis using 2.5% agarose gel with ethidium bromide to see the LMP2A gene band.

ThePCR reaction product was extracted and purified. Sequencing was performed to see variation of base sequence coding LMP2A epitope associated withCTL-HLA A24. Sequencing consists of purification, cycle sequencing and precipitation.

Statistical analysis

The analysis of the LMP2A epitope CTL-HLA A24 gene and its variation wasperformedby descriptive analysis.

RESULTS

Sixty-four subjects were involved in the study and divided into 32 NPC patients and 32 healthy individuals (TABLE 1). Most of NPC patients were in the late stage (Stage IV).

TABLE 1. Characteristics of subjects

Characteristics	NPC patients(%) (n = 32)	Control (%) (n = 32)
Sex		
• Male	21 (65.6)	21 (65.6)
• Female	11 (34.4)	11 (34.4)
Age (year)	50.9 ± 13.9	49.0 ± 14.9
StageTNM		
• I	1 (3.1)	
• II	1 (3.1)	
• III	4 (12.5)	
• IV	26 (81.3)	

Amplification LMP2Agene from peripheral blood sample of NPC patient

Thirty-two peripheral blood samples of NPC patients are amplified according to the prior PCR optimization. DNA band emerged in this experiment only in positive control and there was no emerging band in peripheral blood samples of NPC patients (FIGURE 1A). The amplified of actin gene acted as a control (FIGURE 1B). It showed that none or very few EBVs was taken in the patients' peripheral blood samples. Some of the samples were not good, for the example in sample 10-106, actin was slightly amplified, shown that the quality of this DNA sample was not good comparing to other samples.

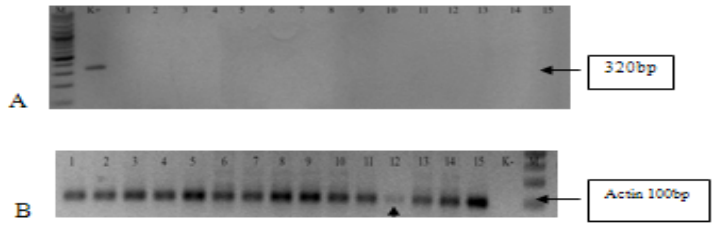

FIGURE 1. (A) PCR Electrophoresis for LMP2A on peripheral blood samples NPC, agarose gel 2%. M is marker 100bp Vivantis, K+ is positive control, number on the upside panel is samples number (1-15). On the right panel is product basepair 320bp, showing by arrow. There is no DNA fragment amplification, (B) PCR of actin gene as acontrol gene, there is fragment amplification in all samples. Showing by headarrow is one of the sample which is too faint DNA amplification

Amplification LMP2Agene from cytobrush sample of NPC patient

Thirty-two cytobrush samples of the NPC patients are amplified according the to the prior PCR optimization. In this test, DNA band emerged only in positive control and most cytobrush samples with variable thickness of the band (FIGURE 2A). For sample number 11-01B, the gene is not amplified, while in PCR actin it is amplified. It is proof that there was not enough even none of EBVs in sample 11-01B so that there was no amplification of LMP2A gene. Likewise, in sample 11-11B, 11-13B, 11-14B and 11-15B, there were no amplified LMP2A gene whereas actin gene (control) were amplified (FIGURE 2B).

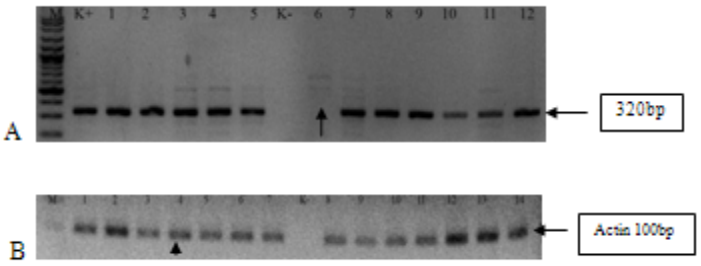

FIGURE 2. (A) PCR Electrophoresis for LMP2A on cytobrush samples NPC, agarose gel 2%. M is marker 100bp Vivantis, K+ is positive control, number on the upside panel is samples number (1-12). On the right panel is product basepair 320bp, showing by arrow. DNA fragment amplification show in most of cytobrush samples. Showing by arrow is sample number 6 is no amplification; (B)PCR of actin gene as acontrol gene, there is fragment amplification in all samples. Showing by headarrow is sample number 6 (11-01).

Amplification LMP2Agene from peripheral blood sample of control

Thirty-two peripheral blood samples of control are amplified according to the previous PCR optimization. DNA band emerged only in positive control and it was not found any DNA band in sample groups (FIGURE 3A). Control gene actin was amplified in all samples (FIGURE 3B). It wasproof that the quality of control DNAs were still good and there was no LMP2A gene in peripheral blood samples of the controls.

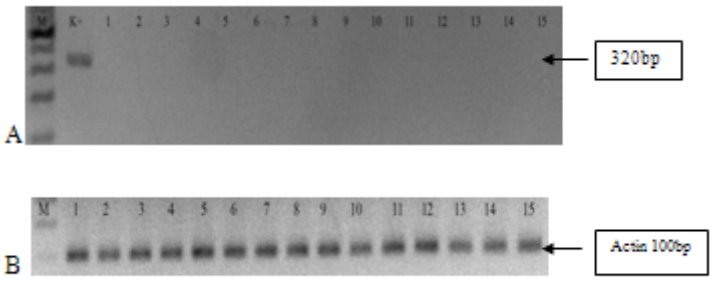

FIGURE 3. (A) PCR Electrophoresis for LMP2A on peripheral blood control group, agarose gel 2%. M is marker 100bp Vivantis, K+ is positive control, number on the upside panel is samples number (1-15). On the right panel is product basepair 320bp, showing by arrow. There is no DNA fragment amplification; (B) PCR of actin gene as acontrol gene, there is fragment amplification in all samples.

Sequence variation of LMP2Agene from cytobrush samples

Twenty six of 32 cytobrush samples were successfully amplified. Only 16 of 26 successfully amplified samples that could be clearly analyzed using sequencing. Based on sequencing test, there was a change in gene sequence compared to wildtype (*Gen Bank* AJ 507799.2). It was also found another epitope besides CTL-HLA-A24, named CTL-HLA-A2, so that the results need to be analyzed thoroughly.

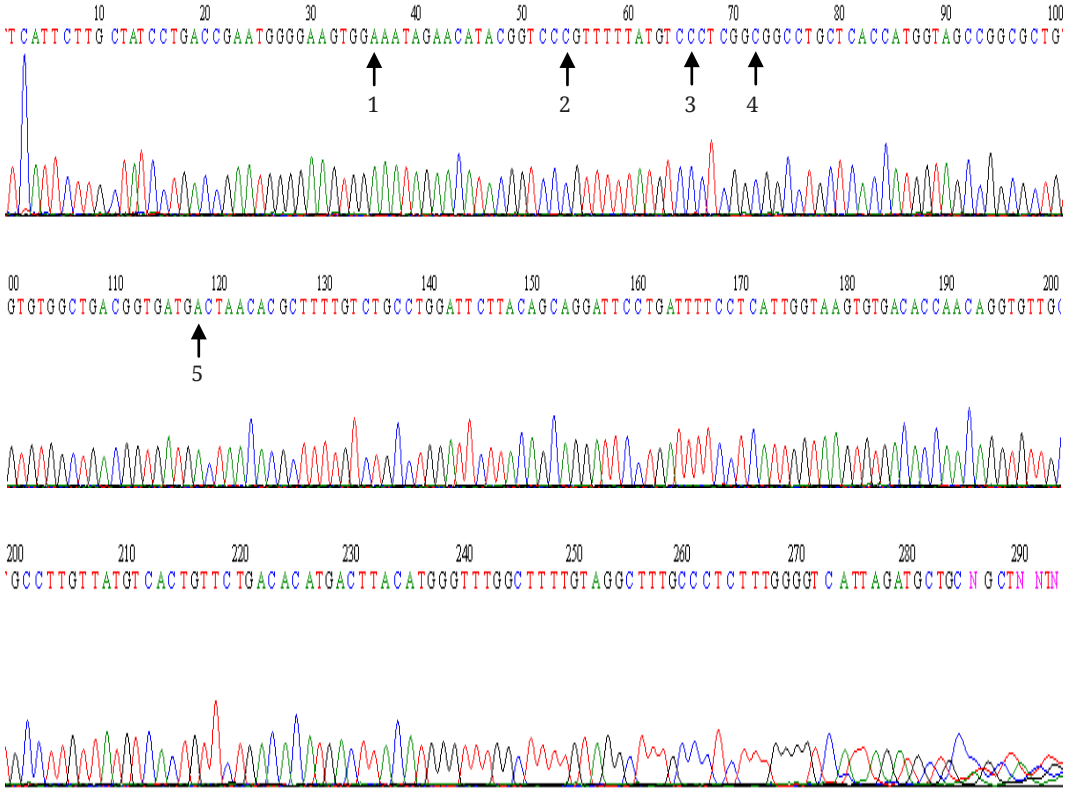

FIGURE 4. The result of variation sequence. Arrow 1 : changes from GGC to GGA; Arrow 2 : changes from CCA to CCC; Arrow 3 : changes from TGC to TCC; Arrow 4 : changes from GGT to GGC; andArrow 5 : changes from TCT to ACT

Distribution Sequence variation of LMP2Agene from cytobrush samples

Based on analysis, it was found five different variations in each sample which undergoing reanalysis to see the distribution between those variations in all samples (TABLE 2). The variations are: Type I: TCT>ACT (change in one nucleotide), Type II: GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC(change in four nucleotides) and Type III: GGC>GGA, CCA>CCC,TGC>TCC, GGT>GGC,TCT>ACT (change in five nucleotides).It was found, Type I with a change in one nucleotide as many as 37.5%, Type II with four changes of nucleotide as many as eight samples (50%) and Type III with five changes of nucleotide as many as 12.5% from all samples.

TABLE 2. Distribution Sequence variation of LMP2A gene from cytobrush samples

Type	n (%)	Sequence variation
Type I	6 (37.5)	TCT>ACT
Type II	8 (50)	GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC
Type III	2 (12.5)	GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC, TCT>ACT
Total	16 (100)	

The relationship between sequence variation of LMP2Agene and tumorstage

From the results above, it was showedthe relationship between sequence variation of LMP2A gene and tumorstage (TABLE 3). In type I, it was found the same presentation between stage 4a and 4c which was 50%. In Type II, the presentation differed between stage3, 4a and 4b whereas the highest presentation was in stage4b (62.5%). In Type III, it was also found the same presentation between stage 4a and 4b which was 50% each. Compared to Type II and Type III, only in Type I (the change of nucleotide TCT>ACT) stage 4c could be found.

TABLE 3. The relationship between sequence variation of LMP2A gene and tumourstage

Sequence variation	Stage [n (%)]					
	1	2	3	4a	4b	4c
Type I (TCT>ACT) (n = 6)	-	-	-	-	3(50)	3(50)
Type II (GGC>GGA; CCA>CCC; TGC>TCC; GGT>GGC) (n=8)	-	-	1(12.5)	2(25)	5(62.5)	-
Type III (GGC>GGA; CCA>CCC; TGC>TCC; GGT>GGC; TCT>ACT) (n = 2)	-	-	-	1(50)	1(50)	-

DISCUSSION

Based on subject characteristic, the numberof male patients wasmore than female patients with the ratio of 2:1. This result wasin line with Lo *et al.*[2] research where male patients tends to be 2-3 times more than female patients. Sun D[1]mentioned that NPC incidence in male patients tends to be higher by 2.5-2.6 times than female patients. In age characteristic, it found that the average of age was50.9. It wasshown that NPC occurrencemainly found in middle ages. Girri and Sarraf[15] suggestedthat the peak of NPC occurrencewasin the 50-59 of age. The unspecified symptoms of NPC (flu-like symptom, laryngitis or nasal inflammation in the early stage of cancer) isthe main reason why most of NPC patients come to hospital at late stage. This study showed thatmost patients (81.3%) at the stage IV of NPC while the stage I was only 3.1%.

The most abundant LMP2A gene couldonly be amplified in cytobrush sample, whereas LMP2A was not expressed in peripheral blood sample

of NPC patients or control gene. It mightoccur because cytobrushwastaken from the site of cancer so the probability of captured EBV-infected B cell wasmuch higher than in peripheral blood samples. For NPC peripheral blood samples, the volume of blood wasonly 100 µL and 900 µL lysis buffer. With only sucha small amount of blood volume, it contributesto the unamplified LMP2A gene because there are other types of cells in the blood sample. In addition, the possibilities werealso caused by very low concentration of DNA in peripheral blood than in cytobrush samples. Junker [16]said that so many years after acute infection of EBV, infected B cell and in latent phase in the peripheral blood accounts for more than 1-60 per 10^6 B cells (± 10 mL blood). Kieff[17] mentioned that EBV that infects B cell is found in only 1 of a million B cells.

According to the result ofsequencing test from cytobrush samples, there was a change in gene sequence compared to wildtype (*Gen Bank* AJ 507799.2). It was also found another epitope besides CTL-HLA-A24, named CTL-HLA-A2, so that the results need to be analyzed thoroughly.

From sequence analysis of LMP2A gene, it was found five nucleotide variations: the change of base sequence from GGC to GGA (C→A in position 1350, *GenBank* AJ 507799.2), however, translated amino acid remained the same (Glycine, G). the second was the sequence change from CCA to CCC (A→C in position 1374, *GenBank*AJ 507799.2), however, translated amino acid remained the same (Proline). The third was the sequence change from TGC to TCC (G→C in position 1375, *GenBank*AJ 507799.2) and transformed cystein to serine. The fourth was the sequence change from GGT to GGC (T→C in position 1392, *GenBank* AJ 507799.2), however, translated amino acid remained the same (Glycine). The fifth was the sequence change from TCT to ACT (T→A in position 1438, *GenBank* AJ 507799.2) and transformed serine to threonine.

Cystein and serine are non-charged amino acid, so that the change from cystein to serin will not affect the whole polarity and charge of amino acid sequence. But another possibility emerges: it may cause the difference of binding action with HLA amino acids. The change of amino acid sequence may cause non optimal recognition of the CTL. Research conductedby Wiqoyah[18] towards three epitopes from lymphoblastoid cell line from NPC patients found that there werechange of amino acid at epitope 8 from epitope TYGPVFMCL, epitope 1 from epitope CLGGLLTMV and epitope 6 from epitope SSCPLSKILL. The difference between our research and Wiqoyah'sisin type and samples amount.

Khanna *et al.*[13] mentioned that the change of amino acid sequence in the anchor epitope CTL will cause nonbinding epitope with its HLA, so that CTL will not recognize HLA-epitope complex. In the other hand, the change of amino acid sequence in the part that binds T cell receptor (TCR) will cause CTL to recognize the epitope but not optimal.Ming Lung *et al.*[19]suggested that the expression of LMP2A in NPCis highly related to the evading mechanism of EBV against immune surveillance represented by CTL.

The 119 amino-terminalcytoplasmic domain of LMP2A coded from the first exon hold an important part in LMP2A function. In that part there is phosphorylation spot for tyrosine, serine and threonine as well asabinding spot for certainprotein kinases such astyrosine kinase from src family coded by Lyn and Fyn, which will later function in inhibiting B cell's signal transduction by interacting with Lyn protein kinase. Tanaka *et al.*[20] statedthat LMP2A is the specific target of CTL, so that the mutation of this target will cause disturbance in CTL response towards LMP2A causing rapid growth of cells in the host body.

Serin amino acid change is reported to be the most common in NPC in China. This change is advantageous forthe EBV to be persistent in B cells. This also showed that LMP2A has an important rolethat causes EBV to be latent in the host.

We found five different variations in each sample which underwentreanalysis to see the distribution between those variations in all samples. The variations are: Type I: TCT>ACT (change in one nucleotide), Type II: GGC>GGA, CCA>CCC, TGC>TCC, GGT>GGC(change in four nucleotides) and Type III: GGC>GGA, CCA>CCC,TGC>TCC, GGT>GGC, TCT>ACT (change in five nucleotides).It can be suggested that Type I with a change in one nucleotide as many as 37.5%, Type II with four changes of nucleotide as many as eight samples (50%) and Type III with five changes of nucleotide as many as 12.5% from all samples.

From the results above, it was obtained the relationship between sequence variation of LMP2A gene and tumourstagewhich are: In type I showed the same presentation between stage4a and 4c which was 50% each. In Type II, the presentation differed between stage3, 4a and 4b whereas the highest presentation was in stage 4b (62.5%). In Type III, it was also found the same presentation between stage4a and 4b which was 50% each. Compared to Type II and Type III, only in Type I (the change of nucleotide TCT>ACT) stage4c could be found. From these results, there was a association between sequence variation and tumorstage. Previous study showed thatdetected EBV in NPC case had a connection with its stage, whereas there was more positive EBVs in late cancer stage. LMP2A are important for the migration of the cell, its increase the metastasis progress. LMP2A mimicry BCR and activated Akt/Pkb from PI3K pathway, the function of pro apoptosis protein Bad and Glycogensintese kinase 3β was inhibited. And its caused β catenin are not degradation, as we know β catenin play important role in cell differentiation and oncogenic transformation. Itwas also a E cadherin component that have function in adhesion of the cell.[21]

In our research, we found another variation compared the previous research conductedby Wiqoyah.[18] The variation is in the upstream sequence of LMP2A from GGC>GGA which is silent mutation and the other variation is in downstream sequence of LMP2A from TCT>ACT which is missense mutation. It is assumed that those sequence variations might be affecting structure change of LMP2A amino acids, so that interaction between LMP2A and CTL epitope is not immunogenic enough to activated CTL, results in escape from immune surveillance. To prove this evidence, further study is needed.

CONCLUSION

It is concluded that cytobrush is still the best choice for screening or diagnosis of NPC. Moreover, the findingof five variations of LMP2A gene which are: the change of base sequence from GGC to GGA, from CCA to CCC, from TGC to TCC, from GGT to GGC, and from TCT to ACT. CCA>CCC and TGC>TCC variation are found on epitope TYGPVMCL which is recognized by HLA-A24 changes into TYGPVFMSL caused by missense mutation. Variation of CCA>CCC does not change the amino acid sequence thus does not alterepitope's shape. Moreover, on epitope associated with HLA-A2 (CLGGLLTMV), there is a change of base sequence from GGT to GGC, however, this change does not transform translated amino acid, it is still glycine (silent mutation). In addition, the new variationsfound in the upstream sequence of LMP2A from GGC>GGA which is silent mutation and the other variation is in downstream sequence of LMP2A from TCT>ACT which is missense mutation.

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