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THE ANALYSIS OF MUTATION PROFILE ON PRE-S1 AND PRE-S2 REGION OF HEPATITIS B VIRUS IN CHRONIC LIVER DISEASE

Citrawati Dyah Kencono Wungu¹, Mochamad Amin², Ulfa Kholili³, Gwenny Ichsan Prabowo¹, Poernomo Boedi Setiawan³, Soetjipto^{1,2}, Retno Handajani^{1,2}

¹Department of Medical Biochemistry, Faculty of Medicine, Universitas Airlangga, Surabaya

²Institute of Tropical Disease, Universitas Airlangga, Surabaya

³Departement of Internal Medicine, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo Hospital, Surabaya

*Corresponding author: cicit.biokimia@gmail.com

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Abstract

Objective

The purpose of this study was to complete the data frequency and mutation profile of Hepatitis B Virus (HBV) pre-S1 and pre-S2 in Indonesia.

Methods

This cross-sectional study was used 32 blood serum samples of Chronic Liver Disease (CLD) patients with Hepatitis B surface antigen (HbsAg) at Endoscopy Outpatient Clinic, RSUD Dr. Soetomo Hospital, Surabaya. Polymerase Chain Reaction (PCR) of HBV DNA was performed on the samples based on pre-S1 and pre-S2 region. Then, electrophoresis was performed on the PCR product and followed by sequencing on samples with positive electrophoresis result. The sequencing results were analyzed by comparing them with the published sequences of HBV nucleotide.

Results

The amplification results of nested PCR DNA HBV with primers based on HBV pre-S1 and pre-S2 region were positive at 21 serums. In patients with CLD in this study, pre-S1 and / or pre-S2 HBV mutations were found in 11 (84.62%) chronically infected HBV patients, 4 (100%) patients with liver cirrhosis, and 4 (100%) HCC patients. Dominant mutations were L101V (16.57%), M120I / T or pre-S2 start codon (10.82%), and F141L (10.81%). M120 and F141L mutations have been previously reported to be associated with CLD, while the dominant L101V mutation in this study as well as several other mutations has not been reported in previous studies.

Conclusions

Mutations of pre-S1 and pre-S2 HBV regions were obtained in 90.48% of CLD patients in the form of substitution and deletion of amino acids.

INTRODUCTION

Hepatitis B virus infection (HBV) is a serious infectious disease that becomes a worldwide concern. According to World Health Organization (WHO) data, it is estimated that more than two billion people worldwide have been infected by HBV. HBV is transmitted through blood, genital secretions, or other body fluids from patients infected with HBV. HBV infections could be acute or chronic. It is estimated that about 780,000 people die annually of chronic liver disease (CLD) due to HBV infection, mainly by cirrhosis of the liver and Hepatocellular Carcinoma (HCC) (1). In

CLD, chronic liver damage occurs due to inflammation, necrosis, fibrosis, and it may progress to carcinogenesis. Clinically called CLD includes chronic hepatitis B, liver cirrhosis, and HCC (2).

According to the WHO classification, Southeast Asia including Indonesia belongs to areas with high HBV endemicity, where in this region an estimated 100 million people live with chronic HBV infection and 70% -90% of HBV-infected populations occur before age 40 (3). The estimation of HBV infected patients in Indonesia are to be 18 million, 10 percent of whom will become liver fibrosis which could then develop into cirrhosis of the liver as well as HCC (4).

Although the pathogenesis of CLD is not fully understood, there are several factors suspected to affect the progression of HBV infection to CLD, one of which is a mutation in the pre-S HBV region (5). In previous studies, there has been a reported association between mutations in the pre-S1 and pre-S2 HBV regions with CLD occurrence (6–9). HBV pre-S1 and pre-S2 regions encode three HBV surface proteins: *Small Hepatitis B surface protein* (S-HBs), *Middle Hepatitis B surface protein* (M-HBs), and *Large Hepatitis B surface protein* (L-HBs); or S (*small*), M (*middle*), dan L (*large*) proteins (10). Pre-S1 and pre-S2 regions are highly immunogenic regions containing many B and T lymphocyte epitopes (11). Besides containing B and T epitopes, pre-S1 and pre-S2 areas also have other functional sites that play important roles in the attachment, replication and progression of viral infections (5).

Mutations in the pre-S1 and pre-S2 regions could lead to the immune escape of the virus as both regions have many epitopes of both B and lymphocytes. Immune escape from virus causes persistence and chronicity of HBV infection. Mutations in the pre-S1 and pre-S2 regions could lead to an imbalance between three HBV surface proteins, Small (S), Middle (M), and Large (L) proteins. The portion of the L protein facing the cytoplasm serves to form the nucleocapsid, while the one facing the ER lumen plays a role in the attachment of HBV with hepatocytes (12). Of the three surface proteins, L protein is only expressed as much as 5-15%, M protein as much as 1-2%, while the rest is S protein (13). Excessive retention of L protein in the endoplasmic reticulum (ER) in the absence of S and M proteins that assist its secretion will cause ER stress and lead to oxidative stress that could trigger liver damage and fibrosis (14,15). ER stress could also activate hepatic carcinogenesis pathways (12).

Data on pre-S1 and pre-S2 HBV mutations in CLD patients in Indonesia is still very limited, thus this research was aimed to analyze pre-S1 and pre-S2 HBV mutation profile in CLD patients in Indonesia.

MATERIALS AND METHODS

Sampling

This study was a cross-sectional study that used a thirty-two (32) blood serum samples obtained from chronically infected HBV patients with a history of positive HBsAg for more than 6 months. Samples were taken at Endoscopy Outpatient Clinic, RSUD Dr. Soetomo Hospital, Surabaya in November, 2015. This study had received approval from the Ethics Committee of RSUD Dr. Soetomo, Surabaya. Informed consent was explained and voluntarily signed by participants before blood sampling. Blood was collected into Venoject tube without anticoagulant. The blood serum was separated, inserted into 3 Eppendorf tubes of 1.5 mL and stored at minus 80°C at the Hepatitis Institute of Tropical Disease (ITD) until being used. Serum HBsAg and ALT were re-examined. Positive samples of HBsAg will be performed PCR on HBV DNA and positive PCR results will be sequenced for analysis of mutations in pre-S1 and pre-S2 regions.

Examination of HBsAg and ALT

ALT was checked with ALT Activity Kit with Ref No.981769 Lot.KB33 from Thermo, Finland by colorimetric enzyme activity assay method, while HBsAg was checked using HBsAg ELISA

Kit with Ref No.1231-1021b010763-01 from Acon, USA by ELISA method.

Viral DNA Extraction and PCR

HBV DNA extraction was performed using QiaAMP DNA Blood Mini Kit with Cat.No.51104 from Qiagen, Germany. The amplification reaction was based on the pre-S1 and pre-S2 HBV regions. Nested PCR DNA HBV in pre-S1 and pre-S2 based gene samples was carried out using a PCR Invitrogen kit with Ref No.25027 from Intron. In the first round reaction PCR used PS-1 (5'-GGGTACCATATTCTTGGGA-3') (2814-2839) and PS-2 (5'- CCCCGCCTGTAACACGAGCA-3') (208-189) primer pair, which, if a negative result obtained, was followed by second-round PCR HBV using PS-3 (5'-TTGGGAACAAGAGACACC-3') (2828-2847) and PS-4 (5'-CACGAGCAGGGGTCCTAGGA-3') (196-177) primer pair (16). PCR was done by using a PCR Thermal Cycler machine: Applied Biosystem Verity 96 Well. The PCR procedure began with the initial denaturation at 94°C for 2 min, followed by PCR cycle with the following steps of 36 cycles: denaturation at 94°C for 60 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 60 seconds. After the 36th cycle a final elongation with temperature 72°C for 7 minutes was performed.

This study also confirmed the presence of positive HBV DNA in samples with HBsAg positive, but still negative with PS-1, PS-2, PS-3, and PS-4 primers. In a sample with a negative HBV DNA, PCR was performed using surface and core regions. The primers used to detect HBV DNA surface region in this study were P7 (256-278): 5'-GTGGTGGACTTCTCTCAATTTTC-3' and P8 (796-776): 5'-CGGTAWAAAGGGACTCAMGAT-3' for first round PCR and HBS1 (455 -474): 5'-CAAGGTATGTTGCCCGTTTG-3' and HBS2 (713-694): 5'-AAAGCCCTGCGAACCACTGA-3' for the second round PCR (17). Primers 12F (3075-3094): 5'-GTGGAGCCCTCAGGCTCAGG-3' and 12R (256-237): 5'-CGAGTCTAGACTCTGTGGTA-3' were also used for amplification of pre-S2 to surface area (18). The primers of core region used to detect the HBV DNA in this study were HBC1 (1650-1669) 5'-TTACATAAGAGGACTCTTGG-3' and HBC2 (2494-2475) 5'-TAAAGCCCAGTAAAGTTCC-3' for first round PCR and HBC3 (1744-1761) 5'-GGGAGGAGATTAGGTAA-3' and HBC4 (2476-2457) 5'-CCCACCTTATGAGTCCAAGG-3' for second round PCR (19).

Detection of PCR products

Electrophoresis was performed using 2% agarose gel run at 100 volts, and then viewed under ultraviolet light and documented using a digital camera. In samples with positive PCR HBV DNA results, purification, labelling, and pro sequencing PCR were performed.

Sequencing

Purification of PCR products was carried out with QiaQuick Gel Extraction Kit Qiagen Cat. #2870. PCR labelling was done with Sequencing Reaction Kit (ABI Prism Big Dye Terminator v1.1 Ready Reaction Cycle Sequencing Kit, Applied Biosystems). DNA Sequencing was performed using ABI Prism 310 Genetic Analyzer (Perkin Elmer) after previously prepared with a given

Big Dye Terminator and SAM solution, heated, incubated in ice, and transferred into a sterile microtube.

Analysis of mutations in the pre-S1 and pre-S2 regions

Genotype and homology analysis with the nuclear BLAST nucleotide program (NCBI) was performed on the nucleotides of this study and the nucleotides used as a comparison. HBV nucleotide sequences from samples were compared with the sequence of HBV nucleotides obtained from international DNA data banks (DDBJ, GenBank, EMBL) according to the genotype result from BLAST. The sequence results were analyzed in the Clone Manager 9 computer software to determine the mutations in the pre-S1 and pre-S2 regions.

RESULTS AND DISCUSSION

A total of 32 serum samples of CLD patients from Endoscopy Outpatient Clinic, Dr. Soetomo Hospital, Surabaya had been collected in this study. All the patients showed a history of positive HBsAg examination in the past for more than six months and until the time of blood sampling for this study, HBsAg was still positive.

This study showed that CLD patients in Endoscopy Outpatient Clinic, Dr. Soetomo Hospital, Surabaya has the youngest age 14 years old and the oldest age 65 years old, with an average age of 46.06 years. Male CLD patients 71.88% (23/32 people) were more than women 28.12% (9/32 people). The mean age of CLD patients in male patients was 46.43 years with the age range of 21 to 65 years, whereas the mean age in women was 45.11 years with the age range of 14 to 65 years. This supports data from previous studies suggesting that HBV-infected men are more likely to be CLD than women (20–23).

In this study, the majority of CLD patients were found to be chronic HBV infection (65.62%), followed by liver cirrhosis (21.88%) and HCC (12.5%). In each CLD group, the male patients outnumbered the female patients. This was in accordance with WHO (2015) data suggesting the incidence of liver cirrhosis and/or HCC in patients with chronic HBV infection by 20-30%. HCC patients were found in small numbers because HCC patients have a low survival rate, which was about 5 months if without getting adequate therapy (24).

Mean age of chronic HBV infected patients was 40.52 years with age range 14 to 65 years, mean age in patients with liver cirrhosis was 55 years with age range 40 to 65 years, mean age in HCC patients was 59,5 years old with age range 49 to 65 years. The results of this study indicated that chronic HBV infected patients had younger mean and range of age than patients with cirrhosis of the liver and HCC. The age difference of chronic HBV infected patients, cirrhosis of the liver, and HCC was due to the progression of hepatitis B disease which takes time to become liver cirrhosis and HCC. Weledji et al (2014) suggests that it generally takes about ten years for HBV infection to develop into chronic, 20 years to become liver cirrhosis, more than 20 years to become HCC, and also it was suggested that patients with cirrhosis of the liver and HCC were commonly found in the range age 50 to 70 years (25).

There were 15.625% of CLD patients who have an ALT increment exceeding 2 times the normal value limit, 9.375% of CLD patients who have an ALT increase of 1 to 2 times the normal value, and as many as 75% of CLD patients have normal ALT. In chronically infected HBV patients, only 5 of 21

individuals had ALT elevations. In patients with cirrhosis of the liver, none had increased ALT. In HCC patients, 3 out of 5 people had ALT elevation. Patients with normal ALT might be in the immune tolerance or immune control phase of the course of HBV infection (26) or in the immune clearance and the immune escape phase where ALT is fluctuating and not always elevated (27).

In the electrophoresis of PCR results of this study, of 32 serum specimens, HBV DNA in PCR product was detected in 21 (65,63%) serum specimens. Nineteen serum specimens gave positive results with PS-1 and PS-2 primers (a 595 bp HBV DNA band), 2 specimens gave positive results with PS-3 and PS-4 primers (569 bp DNA band HBV) whereas 11 specimens gave negative results with both primer pairs, although it had been repeated several times either by using the same primary pair or by pairing PS-1 with PS-4 and PS-3 primers with PS-2. Some electrophoresis results showed a positive result, but dubious HBV DNA band, as it did not match the expected bond length. The sequencing results from some of these doubtful samples proved to be incompatible with the expected sequence. This could be due to a HBV DNA titer that was too low because it was possible that the patient has taken anti-viral drugs that suppressed previous HBV DNA replication. The use of anti-viral drugs as a treatment for HBV infection, either interferon injection or the use of oral nucleoside analogs could suppress HBV DNA replication so that undetectable DNA HBV titer could be considered a marker of treatment efficacy even if HBsAg remains positive (28).

In this study, from samples with negative HBV DNA using PS-1 and PS-2 primers and PS-3 and PS-4, 3 samples with positive HBV DNA were obtained using other primer pairs. One sample was positive with P7-P8 primer pair, another one sample was positive with HBS1-HBS2 primer pair, and one sample was positive by using 12F-12R primer pair. A total of eight samples still gave negative results with the use of 3 sets of primer pairs above.

In samples that gave positive DNA HBV amplification results, sequencing was performed. There was one sample that had not been successfully sequenced with good results although the result of HBV DNA through positive electrophoresis showed the expected band and several repetitions of sequencing had been performed. Mutations were determined by comparing the nucleotide sequences of the pre-S1 and pre-S2 genes obtained in this study with the nucleotides obtained by other researchers and published. Genotype and homology analysis with the nuclear BLAST nucleotide program (NCBI) was performed on the nucleotides of this study and the nucleotides used as a comparison.

Based on the analysis with the BLAST program, all samples had a high homology (99%) with the B3 genotype so that 3 sequences of nucleotide HBV with B3 genotype which had the highest homology with samples to be processed with sample sequence, ie sequence with accession number EU92618 (Indonesia, homology 99 %), GQ924675 (Malaysia, homology 99%), AB219430 (Philippines, 98% homology). In a genotype analysis to determine the standard sequences used by the BLASTN program, it was found that all samples in this study had high homology with HBV genotype B3. Based on the results of other previous researchers, mutations in the form of pre-S1 and pre-S2 deletions were more common in other HBV genotypes (A, C, and D) compared with HBV genotype B (29).

Based on the results of multiple alignments with Clone Manager 9 program (**Figure 1**), from 20 sequenced samples, mutations in pre-S1 and / or pre-S2 regions were obtained in 18

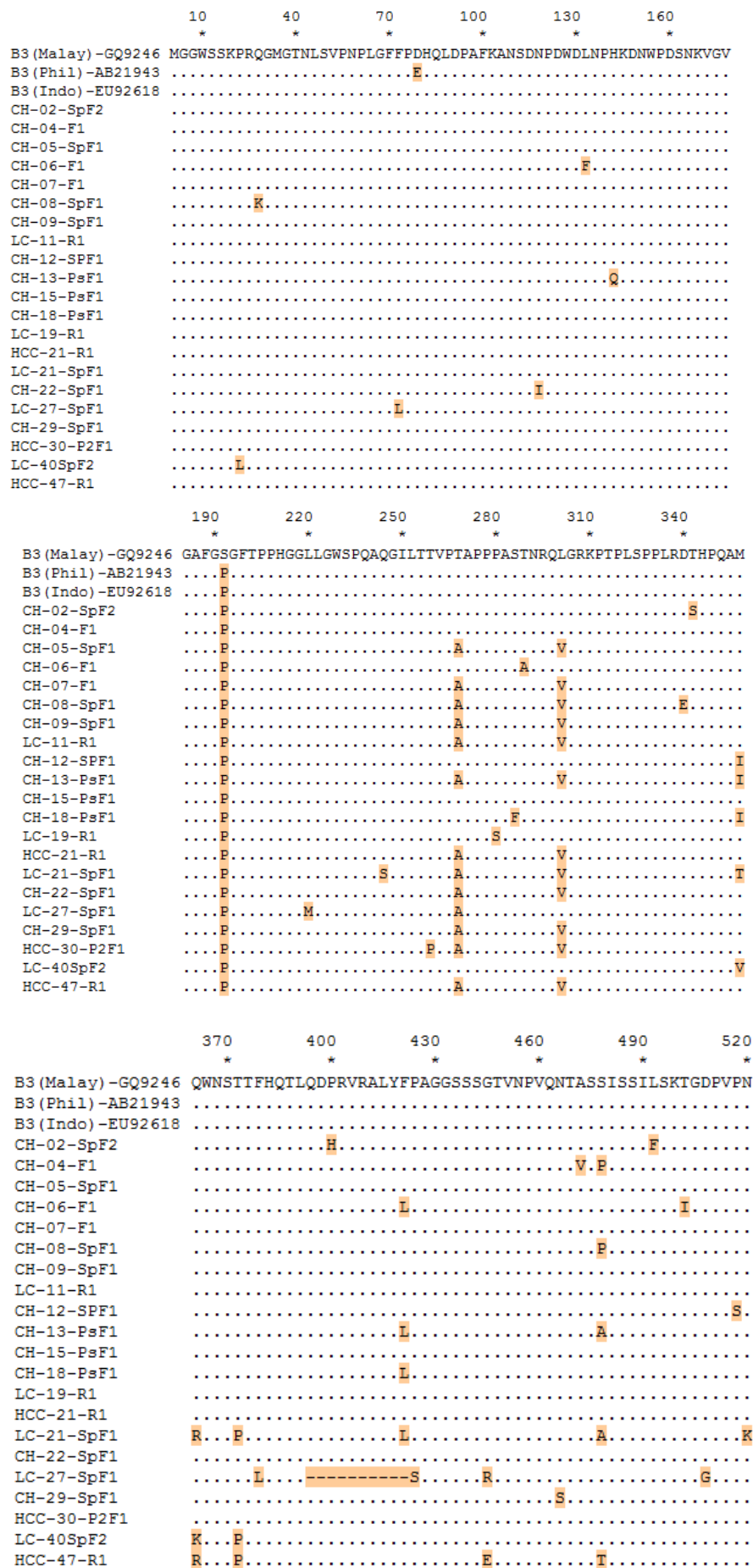


Figure 1. Multiple alignment of HBV pre-S1 and pre-S2 regions using Clone Manager 9 software

samples (90%), primarily in the form of one or two nucleotide substitutions which resulted in amino acid changes. Most of the samples (55.55%) had mutation only in the pre-S1 area, followed by mutations in pre-S1 and pre-S2 (33.33%), and few had mutations in pre-S2 regions only (11.11%). One sample had a mutation of 30 nucleotides (10 amino acids) deletions in the middle of the pre-S2 region. From samples with HBV DNA of positive PCR products and from sequencing results, pre-S1 and / or pre-S2 HBV region mutations were obtained in 84.62% of chronic hepatitis B patients, 100% of liver cirrhosis patients, and 100% of HCC patients. CLD patients with the highest number of mutations were two patients with liver cirrhosis who each had 6 different mutations. HCC patients had 1 to 2 types of mutations in which all three HCC samples in this study all had L101V mutations. In contrast to previous studies (5,16,30), HCC patients in this study had no mutations in the form of deletions, however a mutation of L101V was obtained.

Mapping of mutations in this study showed that all successful sequencing samples had mutations in the epitope of B lymphocytes. Many mutations occurred in epitopes of T lymphocytes and pre-S1 functional areas and / or pre-S2 HBV. Only one mutation (Q82S) occurred in the pre-S functional area without concerning the epitope areas of B and or T lymphocytes. In this study, the affected pre-S functional areas include the Hsc70, CAD, nucleocapsid binding regions, the binding region of the hepatocytes, transactivator domain, S-promoter, pre-S2 start codon, as well as pHSA binding region. This is consistent with previous studies showing that pre-S1 and HBV pre-S2 mutations generally include epitopes against B and T lymphocytes and then trigger mutations in pre-S functional areas (14,31).

The dominant type of mutation in this study was the substitution of Leusin amino acid at position 101 to Valin (L101V) found in 13 of 21 samples of CLD patients (61.9%), followed by substitution of pre-S2 start codons (amino acid 120 position) to threonin or isoleucine (M120I / T) found in 5 of 21 CLD patients (23.8%), and substitution of Amino acid Fenilalanin at 141 to leucine (F141L) found in 4 of 21 CLD patients (19.04 %).

Previous studies have shown that mutations in the pre-S1 and pre-S2 HBV regions are found in many CLD patients (30,32,33). The frequency of pre-S1 and pre-S2 HBV mutations in CLD patients is high in hepatitis B endemic countries (34). Other results suggest that the HBV mutation in the form of amino acid substitution at various nucleotide sites is found up to 75.7% in CLD patients (5). Based on the mapping done by Zhang et al (2015), mutations in the pre-S1 and pre-S2 HBV regions may interfere with the recognition of immune epitopes and cause functional impairment of HBV surface protein when they occur in epitopes against B cells (aa 12-53, aa 72-78, aa 94-117, aa 120-143, and aa 157-167), epitopes against T cells (aa 21-30, aa 52-67, aa 120-145, and aa 163-172), pre-S1 start codons (aa 1), S promoter (nt 3045-3180), CCAAT box binding factor binding site (nt 3137-3141), topological domains covering Hsc70 (aa 74-118) and CAD (aa 81-105) binding sites, nucleocapsid binding sites (aa 103-127), pre-S2 start codons (aa 129), and pHSA (aa 122-135).

Mutations in the pre-S region are found to be deletions, although there are a few researches that suggest mutations in the form of amino acid substitutions at different sites. In a study conducted by Zhang et al (2015) on CLD patients, there were many point mutations in the pre-S1 and / or pre-S2 regions in the form of amino acid substitutions in some codons, ie W4, D27G, N51Q, D54A, V60, A62S, Q100, T125S, R137, S166, and

K167R (5). Mutation at codon 125 in the study was also obtained in this study. A study conducted by Chien et al (2008) mentions a mutation of amino acid substitution at codons 4, 7, and 81 of the pre-S1 region and the pre-S2 start codon in HCC patients (35). In another study conducted by Yin et al (2010) mentioned the substitution of codons 4 and 60 of pre-S1 in CLD patients (36).

In this study, the dominant type of mutation was the substitution of Leusin amino acid at position 101 to Valin (L101V), followed by substitution of pre-S2 start codon (M120I / T), and substitution of amino acid F141L. This type of L101V mutation has not been reported in previous studies. In this study, 100% (3 of 3) patients with HCC had L101V mutations.

In this study, there was only one mutation in the form of pre-S2 deletion of 30 nucleotides in the middle of the pre-S2 region in patients with cirrhosis of the liver. This is consistent with Chen et al's (2006) study showing that in HBV with genotype B, fewer deletion mutations were obtained than genotype C (6). The study conducted by Utama et al (2011) also reported the frequency of pre-S1 deletions and/or pre-S2 was low in CLD patients in Indonesia (30). Based on research conducted by Kim (2014), deletions in the pre-S2 region of HBV are known to play a role in the transition of chronic HBV infection to liver cirrhosis (37). Another study by Su et al (2014) reported that on the dissection of hepatic liver cirrhosis, there was a deletion in the dominant pre-S2 region in the nucleotide region of the 2-57 pre-S2 regions, with or without the M120 mutation. Deletions in the nucleotide region 2-57 pre-S2 region cause decreased synthesis of S and M proteins as well as an increase in L protein causing the formation of Ground Glass Hepatocyte (GGH) in hepatocytes and causing liver fibrosis (38).

In a study conducted by Mun et al (2011), a mutation of F141L was associated with CLD risk and was significantly associated with M120 mutations in the pathogenesis of CLD. F141L and M120 double mutations can enhance the pathogenic ability of HBV and help HBV maintain its viral life cycle. The combination of F141L and M120 has a higher cell proliferation capacity than a single mutation. It is said that F141L mutations did not occur simultaneously with pre-S2 deletions (9). This was in line with the results of this study wherein a sample of liver cirrhosis having a pre-S2 deletion no F141L mutation was found.

The M120 mutation located in the pre-S2 start codon was a frequent mutation in previous studies (12,29,34). Mutations in the pre-S2 start codon may interfere with M protein synthesis, thus impairing the balance of the S, M, and L protein ratios. The decreased M protein leads to increased and retention of the L protein until ER stress arises. This leads to mutations in the pre-S2 start codons reported to be associated with CLD, especially in patients with cirrhosis of the liver (29,39).

Other mutations found in this study were amino acid substitutions in codons 25, 40, 74, 82, 94, 96, 114, 115, 121, 125, 127, 134, 142, and 149. Based on a study conducted by Lin et al (2013), amino acid substitution occurring in the pre-S region might cause changes in the immune target region when it concerns the epitope of lymphocytes B and or T resulting in immune-escape conditions, disturbance of introduction by class II MHC-dependent helper T cells or reduce the affinity of the class I MHC binding on the hepatocyte surface (40). Grotola et al (2002) showed that the number of pre-S2 mutations was associated with recurrence of HBV infection after liver transplantation independent of immunoglobulin prophylaxis (41). Variations of mutations in the pre-S1 and pre-S2 regions might alter the overall conformation of the HBV surface protein, altering the antigenic

properties of HBV against anti-HBs and causing the progression and chronicity of HBV infection (40). Other than that, studies in molecular field show that pre-S mutant interacts with many signalling proteins in cell cycle, cell survival, apoptosis resistance, genetic instability, and cell proliferation (42).

In HBV infection, in the event of mutations that may cause epitope functional disruption of B cells and T lymphocytes, immune escape from the virus might result in HBV replication and further liver damage. Mutations in the epitope region against B and T lymphocytes might also cause further mutations in other regions of pre-S1 and pre-S2 (14,35).

Mutations in Hsc70 or CAD causes L proteins unable to maintain their topological structure, as both Hsc70 and CAD are important determinants for maintaining the double topological structure of protein L (43). This will lead to the retention of L proteins and subvirus particles in the ER due to non-secretion (44).

Disorders in S promoter could suppress S protein production and cause a disruption of the balance ratio between protein S and protein L. Normally, L protein requires S protein to be secreted out of ER. When S protein production decreases, there could be retention of L protein in ER that can cause stress of ER (45,10). It was reported in a previous study by Zhang et al (2015) that point mutations in the S promoter region might cause a reduction in HBV surface protein that leads to intracellular retention of viral envelope proteins and causes ER stress (5).

Nucleocapsid binding regions were important for viral maturation so that virus particles and components could be secreted out of the ER without causing ER stress (16). Mutations in pHSa could cause M-protein synthesis disturbances thereby altering the balance of S, M, and L protein ratios to also cause ER stress (31).

Studies on the incidence of pre-S1 and pre-S2 mutations of HBV in CLD patients in Indonesia were previously performed by Utama et al in 2011. In the study, it was reported that mutations in the form of deletions and insertions in pre-S1 and pre-S2 HBV were only found in 12.1% of CLD patients. The incidence of pre-S1 and pre-S2 HBV mutations in the study was said to be very low when being compared with other Asian countries (30). It was consistent with this study, in which a pre-S2 deletion mutation was found in only 5% of CLD patients. In a study also conducted by Utama et al in 2012, it was reported that the frequency of pre-S2 start codon mutation (M120) was quite high (22%), especially in patients with liver cirrhosis and HCC. In this study, M120I / T mutations were quite frequent (20%) but not found in HCC. Other mutations reported in Utama et al (2012) were T130 (26.1%), Q132 (28.7%), and A138 (29.5%) (46).

There were several factors that affect the progression of HBV to CLD, including host factors, viral factors, and other factors. Host factors include age, sex, and immune status. Viral factors include HBV genotype, viral load, pre-S mutation, and T1762 / A1764 mutations. Other factors include co-infection, aflatoxin, cigarette smoke, and alcohol. Virus factors could mutually influence each other so that there could be different mutation patterns between genotypes or double mutation which have the different influence to the incidence of CLD (47).

So far, studies on pre-S1 and pre-S2 mutations of HBV in CLD patients in Indonesia were still limited and no research had been found in other regions in Indonesia, so there was still possibility of genotypic distribution in Indonesia causing various frequency and types of different pre-S1 and pre-S2 HBV mutations in different regions.

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