THE EXISTENCE OF GENE DEOXYRIBONUCLEIC ACID POLYMERASE MUTATION FROM POSITIVE HEPATITIS B SAMPLES IN BANDUNG, INDONESIA

TINA ROSTINAWATI1*, DEBBIE SOFIE RETNONINGRUM2, DESI PRATIWI3
1Department of Biological Pharmacy, Pharmaceutics, Faculty of Pharmacy, Universitas Padjadjaran, Indonesia. 2Department of Biological Pharmacy, Pharmaceutics, School of Pharmacy, Institut Teknologi Bandung, Indonesia. Email: t_rostinawati@unpad.ac.id

ABSTRACT

Objective: The objective of the study is to complete mutation existence in reverse transcriptase (RT) domain of the viral polymerase.

Methods: The study was done by amplification step of viral polymerase gene fragment, agarose gel electrophoresis, polymerase chain reaction product purification with GFX column kit, sequencing, and sequencing result analysis. The samples were derived from clinical laboratory in Bandung Indonesia.

Results: The result showed mutations in deoxyribonucleic acid (DNA) fragment encoding for RT domain viral polymerase in sample 6, 7, and 8. There were mutations leading to amino acid substitution L526S in sample 6, D551E in sample 7, and D552E in sample 8.

Conclusion: D551E and D552E substitution occurred in YMDD motif RT DNA polymerase that produced YMDE mutant. L526S, D551E, and D552E were estimated as antivirus-resistance mutants that have never been reported before.

Keywords: Hepatitis B virus, Mutation, Gene encoding for reverse transcriptase deoxyribonucleic acid polymerase.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major human health problem with a mortality rate of 1.2 million deaths per year worldwide due to this disease. HBV infection is characterized by a high rate of development of chronic infection with either high- or low-titer viremia. HBV infection causes acute and chronic liver disease, including chronic hepatitis, cirrhosis, and liver cancer (hepatocellular carcinoma) [1,2].

Various attempts have been made to suppress the development of HBV infections, including antiviral drug development and development of an HBV vaccine, but there is no effective antiviral treatment for patients chronically infected with HBV. Treatment with interferon is quite beneficial for most patients infected with chronic HBV but is limited to an overall average response of <40% since dosages of interferon are usually limited because of side effects.

Replication of HBV deoxyribonucleic acid (DNA) takes place through reverse transcription of intermediate ribonucleic acid pre-genomically. It is the basis for the development of nucleoside analogs that inhibit the activity of HBV reverse transcriptase (RT), such as lamivudine, famciclovir, and others as an alternative to interferon therapy [1-3].

Lamivudine has been shown to suppress HBV replication and has few side effects and is used to treat chronic HBV infection. Lamivudine can also prevent reinfection after liver transplantation. The use of lamivudine for long-term treatment is reported to cause the occurrence of viral resistance. In 14% of patients treated with lamivudine for 1 year, an amino acid substitution occurred in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the C domain of HBV DNA polymerase (HDP) at codon 552, either methionine into valine (M552V) or methionine into isoleucine (M552I). Another amino acid substitution in B domain of HDP, i.e., the substitution of leucine into methionine at codon 528 (L528M), was also reported to be coincident with the amino acid substitution in the YMDD motif. L528M amino acid substitutions are common in patients treated with famciclovir [1,2,4].

In addition to mutations in the gene encoding the HDP region that result in viral resistance, HBV may also have mutations in the gene encoding a surface antigen (HBsAg) protein that gives rise to the phenomenon of vaccine-escape mutants. Vaccine-escape mutants are not recognized by any anti-HBsAg antibodies, so it can infect people who have been vaccinated. A study showed that vaccine-escape mutants had been found in clinical isolate samples from four cities in Java, Indonesia. Vaccine-escape mutants were derived from substitutions originally reported from Yogyakarta (M133T) and Jember (G145R), as well as a nucleotide substitution/insertion that was seen in samples from Jakarta (at codon 117 and the insertion between codons 136 and 137) (data not reported).

Information about mutations in the genes encoding HDP and HBsAg thus becomes one of the important factors in the treatment and prevention of HBV infection. The existence of HBV mutants can be determined by analysis of the HBV nucleotide sequence. Determination of nucleotide sequences was performed using polymerase chain reaction (PCR), a technique for replicating DNA in vitro. PCR is used to amplify specific regions of DNA, which can be a single gene, part of a gene, or a non-coding sequence. Primers to initiate the PCR reaction are generally short fragments (20-30 bp) of DNA [5].

METHODS

The ethical committee approval was provided by Medical Faculty of Universitas Padjadjaran, and 12 participants were included in this study.

HBV isolates

Samples that used in this research were HBV isolates. HBV isolates were obtained from the main clinical laboratory Paramita Bandung branch, Indonesia to be used as HBV templates for gene amplification. The HBV templates used were HBV DNAs isolated from plasma of chronic Hepatitis B patients through a quantitative HBV PCR examination conducted by the clinical laboratory Paramita Bandung branch. HBV DNA templates had high titer (Table 1).
Amplification of genes encoding HDP fragment
DNA samples were amplified in a 96-well thermal cycler instrument with PCR conditions as follows: Initial denaturation at 94°C for 5 minutes; a reaction cycle consisting of denaturation at 94°C for 1 minute, annealing at 47°C for 1 minute, and elongation at 72°C for 1 minute. Amplification was performed for 35 cycles, and elongation for the last cycle was at 72°C for 10 minutes. The primers used were BCfwd 5’-ACC CCT GCT CAA GGA ACC TC3’ and polymerase BCrev 5’-AGA TGT TGT ACA GAC TTG GC3’. These primer pairs can amplify fragments of gene encoding RT HDP, which includes codons that encode YMDD motif. Up to 5 ml of a sample of HBV isolate was mixed with Taq buffer solution (1.5 mM MgCl₂, Tris [pH 8.3] 10 mM, 50 mM KCl, and gelatin 200 mg/ml), 200 μM dNTPs, Taq polymerase 5U, 1 ml of 30 pmol BCfwd primer, 1 ml of 30 pmol BCrev primer, and 25 mM MgCl₂, then dH₂O was added to bring to 25 ml.

Purification of PCR product
An I Illustra GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience, Freiburg, Germany) was used to purify the resultant PCR product. Briefly, DNA fragment that was in the gel predefined weight was added capture buffer with volume as much as 10 ml to 10 mg. This mixture was incubated at 60°C until the gel was dissolved then was centrifuged at 3000-4000 rpm for 30 seconds. The dissolved sample was inserted into the GFX column, incubated for 1 minute at room temperature, then was centrifuged at 12,000 rpm for 1 minute. The filtrate in collection tube was discarded. A total of 500 ml of wash buffer was added to the column, incubated, and then centrifuged back. The filtrate in the collection tube was again discarded, and the GFX column inserted into a sterile 1.5 ml Eppendorf tube, to which was added 50 ml of elution buffer, then incubated for 1 minute at room temperature and centrifuged at 5000 rpm to obtain purified PCR product.

Determination of nucleotide sequence
Nucleotide sequence determination was carried out by the Sanger dyeoxy method. A sequencing primer has the ability to determine the nucleotide sequence of sample from 5’ to 3’. Primer sequencing used BCfwd 5’-ACC CCT GCT CAA GGA ACC TC3’ and BCrev 5’-AGA TGT TGT ACA GAC TTG GC3’. PCR products were purified with sequencing primer, Taq buffer, Taq Polymerase, dNTPs, ddNTPs, and stop solution (95% formamide; 20 mM EDTA pH 8.0; 0.05% bromophenol blue, 0.05% xylene silanol FF). Determination of the nucleotide sequence of sample from 5’ to 3’ was performed using an automatic DNA sequencer.

Analysis of nucleotide sequence
The results of nucleotide sequence determination were analyzed by aligning the nucleotide sequence obtained with the nucleotide sequence encoding the HDP gene fragment in the GenBank. These samples were also analyzed with the nucleotide sequence fragments of the genes encoding HBsAg subtype adw, adz, ayr and ayr in the GenBank. Amino acid determination was also carried out by the deduction method, manually comparing the results with the partial amino acid sequence of the RT DNA polymerase and the protein S - HBsAg. Genotype isolates were determined by comparing the results of the determination of nucleotide sequences homologous to the nucleotide sequence of data obtained from GenBank, using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) [6].

RESULTS AND DISCUSSION
Amplification of gene encoding the HDP fragment
The gene fragment encoding HDP was amplified using BCfwd and BCrev primers, with size of the PCR product ±240 bp. PCR products showed the DNA bands between 200 bp and 300 bp of the marker. Gene fragments from all of the isolate samples were successfully amplified, with DNA band size also ±240 bp. The results of electrophoresis of PCR products can be seen in Fig. 1.

Determination of nucleotide sequence
To determine nucleotide sequence, only nine samples were sequenced; three samples were insufficient concentration (data not shown) for nucleotide sequence determination (data not shown).

Analysis of nucleotide sequence
BLAST results showed that the homology of nine samples were 90-99% based GeneBank database (Table 2). Samples with code 2B, 4, 6A, 6B, 7, 12, 20, and 21 showed the nucleotide sequence of the gene encoding HDP genotype B, while sample with code 14 showed 99% homology with genotype E. Genotype differences were based on 8% intergroup diversity of the entire genome or 4.1% diversity of gene encoding surface protein. Genotype B is one of the eight genotypes of HBV spread across Asia, including Indonesia, while the E genotype is more common in the African region [7].

The alignment results showed that three samples (samples with code 7, 12, and 14) had changed in their HDP nucleotide sequences. The results of the alignment of the HDP sequences can be seen in Fig. 2. HDP also has reverse transcription activity and thus does not have a proofreading activity, allowing the occurrence of mutations with high frequency of between 10⁻³ to 10⁻⁶ per nucleotide per day. The result is accumulation of compounded nucleotide sequence variations, some of which can occur during antiviral therapy. In addition, fluctuations in the concentration of intracellular dNTP causes hypermutation, such as [dTTP]/[dCTP] changes G to A [7].

Since mutations to the nucleotide sequence may or may not cause changes in amino acids composition in the resultant protein, the alignment process was also performed on the HDP amino acid sequence. The nucleotide sequences coding genes of HDP and nine samples were translated into amino acids using DNA translate tool (https://www.expasy.org). Alignment of amino acid sequences of HBV isolates samples to the wild-type HBV sample were also done using the BLAST program of NCBI site. The result of the amino acid alignment of nine samples shown in Fig. 3.

Sample with code 7 showed a nucleotide sequence change at position 1577, a changed of a T (thymine) to a C (cytosine), which caused an

Table 1: HBV isolate sample

<table>
<thead>
<tr>
<th>No</th>
<th>Sample code</th>
<th>Titer (UI/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2A</td>
<td>1.1×10⁶</td>
</tr>
<tr>
<td>2</td>
<td>2B</td>
<td>3.2×10⁶</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1.7×10⁷</td>
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<tr>
<td>4</td>
<td>6A</td>
<td>6.8×10⁶</td>
</tr>
<tr>
<td>5</td>
<td>6B</td>
<td>1.9×10⁶</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>3.1×10⁶</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>3.8×10⁷</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>3.5×10⁷</td>
</tr>
<tr>
<td>9</td>
<td>17</td>
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<td>1.0×10⁷</td>
</tr>
<tr>
<td>12</td>
<td>19M</td>
<td>1.6×10⁷</td>
</tr>
</tbody>
</table>

HBV: Hepatitis B virus
HBV: Hepatitis B virus, BLAST: Basic Local Alignment Search Tool

Table 2: BLAST results of nucleotide sequence samples

<table>
<thead>
<tr>
<th>Sample code</th>
<th>BLAST result (Result identity)</th>
<th>GeneBank Access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>4</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>6A</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>6B</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>7</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>12</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>14</td>
<td>HBV genotype E</td>
<td>X75657</td>
</tr>
<tr>
<td>20</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
<tr>
<td>21</td>
<td>HBV genotype B</td>
<td>X98077</td>
</tr>
</tbody>
</table>

HBsAg in the HBV vaccine [10,11].

Analysis of sample 12 showed a change in nucleotide sequence at five positions, i.e., T1590C, C1611T, T1653G, A1659T, and T1674C. The mutations at position 1590, 1611, 1659, and 1674 were not accompanied by amino acid changes, so these mutations were silent. The change of thymine (T) to guanine (G) at position 1653 caused aspartic acid (D) to change to glutamic acid (E) at codon 551, in the gene encoding HDP. Both amino acids have similar properties, both are acidic and polar. The difference is that glutamic acid has one more carbon atom than aspartic acid, such that glutamic acid is slightly more non-polar. This mutation changed the YMDD motif in HDP to YMDE. The YMDD motif is catalytic for the RT activity of DNA polymerase, involved in nucleotide binding to the viral replication process. It has been widely reported that mutations in the YMDD motif will change the activity of RT thereby reducing the ability of the mutant to replicate. However, some amino acid substitutions in the YMDD motif are capable of inducing resistance to the nucleoside analog lamivudine. Lamivudine is a cytidine analog that effectively inhibits viral replication by suppressing the activity of RT through its action as a chain terminator. Mutations in the YMDD motif alter the conformation of the catalytic site of RT activity. The glutamic acid molecule is bigger and acts as a steric hindrance thus inhibiting the binding of lamivudine at the RT nucleotide binding site [12-14]. The substitution YMDE was also seen in sample 14 at codon 552; the different position of this codon is due to genotypic difference in genome length. Furthermore, homology model of HDP structure is not done because sequence homology between HDP and HIV-1 RT, usually used as a template for HDP, is less from 30% [15]. Hence, binding affinity between L526S, D551E, and D552E of HDP can be predicted.

These L526M, D551E, and D552E amino acid substitutions seen here have not been previously reported. Various YMDD variants have been reported, i.e., YIDD, YVDD, YADD, YLDD, YKDD, YRDD, and YTDD [12].

CONCLUSION

Amino acid substitutions, i.e., L526M, D551E, and D552E were in areas of the HB DNA polymerase protein with RT activity, especially the YMDD motif (D551E and D552E), which are important for viral replication. The location of these mutations, the active site of RT activity, is an important target in the treatment of hepatitis B; thus, mutations that occur in this area can reduce the sensitivity of the virus to nucleoside analogs and lead to the development of resistance to these antiviral drugs.

ACKNOWLEDGMENT

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REFERENCES