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MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

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HEPATITIS B VIRUS X GENE MUTATION WITH PREDOMINANCE A1762T+G1764A DOUBLE MUTATION ON CHRONIC LIVER DISEASE PATIENTS IN SURABAYA, INDONESIA

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History

Received: 19th March 2019
Accepted: 5th April 2019

Keywords:

Hepatitis B, Chronic Liver Disease, X gene, mutation

Abstract

Background: HBV infection is a major problem worldwide, especially in developing countries including Indonesia. Mutations in the HBV gene X are commonly found in patients with CLD, especially in cirrhosis and hepatocellular carcinoma. Mutations in the X gene can cause loss of stability, increased transactivation function, and decreased anti-apoptotic ability of HBx protein.

Aim: The aim of this study was to detect HBV X gene mutations in CLD patients in Surabaya.

Methods: This was a cross sectional research taking samples at Dr. Soetomo General Hospital, Surabaya, Indonesia. This study used nested PCR by targeting HBV X gene. Samples showing positive HBV DNA PCR results were followed by sequencing and X gene mutation analysis by comparing sequencing results with reference strains.

Results: In this study, 30 samples of CLD patients with positive HBsAg in Dr. Soetomo Surabaya were obtained. From the results of the multiple alignments, 12/30 samples (40%) had mutations on HBV X region which overlapped with Core Promoter region. There were 3 types of substitution mutations on HBV X gene (C1632T, T1753A/C/G, A1762T, and G1764A) with the dominant mutation types were A1762T and G1764A mutations, in which both mutations were found together as double mutation.

Conclusion: X gene mutations were found in 40% CLD patients in Surabaya with the dominant mutation was in the form of double mutation A1762T and G1764A in 30% CLD patients in this study. The mutation was found mostly in advance stage of CLD.

INTRODUCTION

Around 240 million people worldwide suffer from chronic hepatitis B virus (HBV) infection and around 600,000 people die each year from HBV infection and Hepatocellular Carcinoma (HCC) [1]. HBV infection is a life-threatening infection, as besides of causing chronic liver disease, this virus can also cause cirrhosis and HCC which potentially leads to death [2]. The prevalence of chronic HBV infection in Indonesia based on data from the Ministry of Health of the Republic of Indonesia is around 8%. About 50% of these have liver disease that has the potential to lead to Chronic Liver Disease (CLD) [3].

Although the mechanism of the occurrence of CLD is not fully understood, one of the factors suspected to play a role is

the Hepatitis B X antigen (HbxAg) protein encoded by HBV which is encoded by X gene. HBV X gene can integrate directly into host DNA in many places and affect the transcription of genes in host cells through various signaling pathways, such as Ras, NF- κ B, activating protein-1 (AP-1), and janus kinase (JAK / STAT) [4]. Gen X is the smallest ORF encoding HBx. HBx protein is a non-structural multifunctional protein of 465 bp (nt 1374-1838) or 154 amino acids with a molecular weight of 16.5 kDa containing important elements such as EnhII (nt 1636-1744), core promoter (nt 1613-1849), and micro RNA binding area (nt 1362-1383). This causes the HBx mutation to affect these areas as well [5]. HBx protein is so named because the sequence of X genes is not homologous with various known proteins [6].

Mutations in the HBV gene X are commonly found in patients with CLD, especially in cirrhosis and HCC. Protein X

HBV is multifunctional and can affect gene transcription, signaling pathways, cell stress response, cell cycle control, viral replication, and apoptosis [7]. X gene mutations, especially in the terminal C, are often found in CLD because this region has an important role in cell growth, proliferation, transformation and transactivation [8]. Gen X also overlaps with several other important HBV genes that are important for cell replication [9]. Data regarding the prevalence of HBV gene mutations in CLD patients in Indonesia, especially Surabaya, are still unknown. The results of this study are expected to contribute to further research and prevention efforts suitable for CLD patients in Indonesia.

MATERIALS AND METHODS

Sampling

This research was a descriptive cross-sectional study that took samples of chronic liver disease patients with positive HBsAg who visited the Internal Medicine Unit of Dr. Soetomo General Hospital, Surabaya in 2017. The inclusion criterias in this study were: Chronic hepatitis B patients with a history of positive HBsAg \geq 6 months, had diagnosis from internist as chronic hepatitis/liver cirrhosis, or hepatocellular carcinoma, volunteered to participate in the study and signed informed consent. Exclusion criterias in this study were: Patients coinfectd with HCV or HIV, and received immunosuppressant therapy. The study was conducted after obtaining approval from the Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya (No 513/Panke. KKE/ VIII/ 2017). Blood collection was taken from cubital vein with 4 mL of blood put on a venoject tube with EDTA. Blood samples were then taken to the Laboratory of Hepatitis Institute of Tropical Disease (ITD) of Universitas Airlangga for laboratory examination. Serum separation from the blood was performed by centrifugation.

HBV DNA Extraction

HBV DNA was extracted using the QIAamp DNA Extraction kit (Qiagen, Inc., Hilden, Germany) with Cat.No.51104 using procedures in accordance to the kit. Controls were treated as the same as the sample.

PCR X Gene

A total of 5 μ L DNA was used for amplification by nested PCR technique, using a PCR 2x PCR Master mix solution (iNtRON®) kit with Ref No.25027. The PCR process for X gene was carried out using: outer primers: XF1 (forward): 5-CATGCGTGGAACCTTTGTG-3 (nt:1233-1251) and XR1 (reverse): 5-CTTGCCTKAGTGCTGTATGG-3 (nt: 2072-2053), inner primers: XF2 (forward): 5-TCCTCTGCCGATCCATACTG-3 (nt: 1254-1263) and XR2 (reverse): 5-CAGAAGCTCCAAATTCTTATA-3 (nt: 1937-1916) [10]. Reaction mixture was made in 0.2 mL eppendorf tube with total volume of 50 μ L for 1 sample. PCR was performed using the DNA thermal cycler: Applied Biosystem Veriti 96 Well. For first round PCR, in the initial stage an initial denaturation was performed with 94°C for 5 min, followed by 35 PCR cycles: denaturation at 94°C for 50 s, annealing at 50°C for 50 seconds and elongation at 72°C for 60 seconds. At the end of the process, the final extension is done at 72°C for 7 minutes. For second round PCR, at the initial stage an initial denaturation was performed at 94°C for 5 minutes, followed by 35 PCR cycles with the following details: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 45

seconds. At the end of the process, the final extension is done at 72°C for 7 minutes. Second round PCR was performed when first round PCR gave negative result. Nested PCR technique was used in order to increase sensitivity and specificity of PCR. When nested PCR procedure above still gave negative result, following primers were used for another nested PCR procedure: XproF1 (forward): 5'-CTCTGCCAAGTGTGTTGCTGA-3' (nt 1171-1190) and XproR1 (reverse): 5'-CAAGGCACAGCTTGGAGGCT-3' (nt 1886-1867) inner primers: XproF2 (forward): 5'-TTGCTCGCAGCCGGTCTGGA-3' (nt 1295-1314) and XproR2 (reverse): 5'-TGAACAGTAGGACATGAACA-3' (nt 1847-1866). For both PCR rounds, at the initial stage an initial denaturation was performed at 94°C for 2 minutes, followed by 30 cycles with the following details: denaturation 94°C for 1 minute, annealing at 54°C for 45 seconds, and elongation at 72°C for 1 minute. At the end of the process, the final extension is done at 72°C for 10 minutes [11].

Detection of PCR Products with Electrophoresis

PCR product was examined by electrophoresis using 2% agarose gel which indicated the expected band, ie 839 bp for first round PCR and 683 bp for second round PCR. 100bp ladder marker, the negative control, and the samples were put into agarose gel. Electrophoresis results were visualized in the UV light and documented. 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 X gene mutations

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 X regions.

RESULTS AND DISCUSSION

In this study, 30 samples from CLD patients with HBV infection were obtained from outpatient / inpatient clinic of Internal Medicine Unit, Dr. Soetomo General Hospital, Surabaya (**Table 1**). All patients showed a history of positive HBsAg examination. Most patients (76.67%) were male. The results of this study are in accordance with several previous studies which stated that patients with CLD and HBV infection in men were more prevalent than women [12]. The cause of the more prevalent male CLD patients can be caused by the influence of hormonal factors. Male sex hormones, namely androgens can increase HBV genome transcription. This HBV genome transcription can cause an increase in the progression of HBV infection to CLD [13].

Table 2. Mutation profile of X gene in CLD patients in this study

Number	Amino Acid Mutations	No. of samples with mutation (n=30)	Functional Regions Affected
1	C1632T	3 (10%)	Core promoter, EnhII
2	T1753A/C/G	7 (15.9%)	BH3-like motif, core promoter, NRE
3	A1762T	9 (30%)	BH3-like motif, core promoter
4	G1764A	9 (30%)	BH3-like motif, core promoter

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 X gene mutations. Other factors include co-infection, aflatoxin, cigarette smoke, and alcohol. Viral 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 [21].

In Indonesia itself, research about HBV X gene mutation was still limited. The number of A1762T/G1764A in this study was higher than previous studies. Artarini et al (2016) had HBx mutant detection in 44 samples with HBV infection and found T1753A/C/G (4.5%), and A1762T/G1764AI mutations as much as 4.5% and 13.6% respectively. However, the clinical status of the patients was unknown [22]. Another study by Fatimawali & Kepel (2014) on 10 samples with acute HBV infection did not find any A1762T/G1764AI mutation. Instead of low number of samples, mutations found in that study did not have any correlation with HBV severity in previous studies [23]. So far, studies on HBV X gene mutations on 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 X mutations in different regions.

ACKNOWLEDGEMENT

Our thanks go to Universitas Airlangga and The Ministry of Research, Technology, and Higher Education for the funding of this research. Also, we thank CLD patients who had contributed to give blood in this study. Thank you also to the staffs at Internal Medicine Department in Dr.Soetomo General Hospital, Surabaya and Institute of Tropical Disease (ITD) which had helped the course of research, and various parties involved in this research that we could do not mention one by one.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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