

Gene X Two Triple Mutations Predominance on Chronic Hepatitis B Virus in Padang, West Sumatra, Indonesia

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ABSTRACT

Background: Chronic hepatitis B (CHB) is a liver inflammation that lasted more than 6 months and it is influenced by Gene X and viral genotype. Mutations in the Gene X are suspected to having a role in disease progression. This study attempted to detect Gene X mutation and phylogeny from Padang samples of asymptomatic CHB, West Sumatera, Indonesia.

Method: This research took asymptomatic 38 sera positive hepatitis B surface antigen (HBsAg) donors at the Red Cross Center (Padang, Indonesia). The inclusion criteria in this study was regular donor (at least 4 times in a year) that recorded has persistent HBsAg. Exclusion criteria in this study was sample coinfecting with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) and poor quality specimen. Nested polymerase chain reaction (PCR) will detect and amplify the virus genetic continued with X sequence database aligning and mutation analysis.

Results: We discover all the samples were having nucleotide mutation. Of various mutations, we observed the presence of known liver cirrhosis and hepatocellular carcinoma (HCC) -related hepatitis B x (HBx) protein mutant i.e double mutations (HBx130 and HBx131) and two triple mutations (HBx5/HBx130/HBx131) and (HBx127/HBx130/HBx131) were 61% dan 55%. The analysis also showed main genotype is C at 72.2% and followed by B at 27.8%.

Conclusion: We conclude that all the samples already have nucleotide mutations. This study implies that molecular progression has occurred between the virus and the host at asymptomatic CHB.

Keywords: hepatitis B virus, gene x, mutation

ABSTRAK

Latar belakang: Chronic hepatitis B (CHB) merupakan kondisi peradangan hati lebih dari 6 bulan dan dipengaruhi oleh Gen X dan genotip virus. Mutasi pada Gen X dicurigai memiliki peran dalam perburukan penyakit. Penelitian ini bertujuan untuk mendeteksi mutasi Gen X dan filogeni pada asimtomatik CHB di Kota Padang, Sumatera Barat, Indonesia.

Metode: Penelitian ini dilakukan pada 38 serum positif hepatitis B surface antigen (HBsAg) dari Palang Merah Indonesia (Padang, Indonesia). Kriteria inklusi yaitu serum pendonor regular (minimal 4 kali selama 1 tahun) dengan HBsAg persisten. Kriteria eksklusif ialah sampel dengan koinfeksi hepatitis C virus (HCV) atau human immunodeficiency virus (HIV) dan kondisi sampel yang buruk. Polymerase Chain Reaction (PCR) nested akan mendeteksi dan mengamplifikasi genetik virus kemudian dilanjutkan dengan sekuensing serta analisis mutasi.

Hasil: Studi ini menunjukkan semua sampel memiliki mutasi nukleotida. Dari semua mutasi, ditemukan mutant terkait sirosis hepatis dan hepatoselular karsinoma (HCC) hepatitis B x (HBx) protein mutant berupa mutasi doublet (HBx130 dan HBx131) dan dua triple mutasi (HBx5/HBx130/HBx131) dan (HBx127/HBx130/HBx131) yaitu 61% dan 55%. Penelitian ini juga menunjukkan genotip terbanyak ialah C sebanyak 72,2% dan B sebanyak 27,8%.

Simpulan: semua sampel telah memiliki mutasi. Penelitian ini menunjukkan telah terjadi progresi tingkat molekular antara virus dan host pada kondisi asimtomatik CHB.

Kata kunci: hepatitis B virus, gen x, mutasi

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem with more than 257 million people infected worldwide.¹ HBV is a member of hepadnaeviridae family and classified into ten genotypes, i.e genotype A-J. The phylogenetic classification based on more than 8% divergency intergenotype and less than 4% intragenotype HBV genome has a higher mutation rates, about $1.4\text{--}3.2 \times 10^{-5}$ base substitutions per site per year due to the viral transcriptase's lack of proof reading capabilities.^{2,3} In Indonesia, genotypes B and C predominate.⁴ When compared to HBV genotype B, HBV genotype C is known to induce severe liver disease, cirrhosis, and HCC.⁵ The viral genome is 3200 kb in length and contains partly double-stranded circular deoxyribonucleic acid (DNA) with overlapping open reading frames (ORFs): envelope (S/Pre S), core (C/Pre C), polymerase (Pol) and X (HBx) proteins.^{6,7} The HBx protein is a multifunctional protein of 154 amino acids. The Gene X's genetic variability comprises genotypic specific variation as well as mutations that emerge after chronic infection.^{8,9} Its coding sequence coincides with key viral replication areas, including the basal core promoter (BCP). Thus, a mutation in the Gene X can influence other genes and change HBV expression in addition to causing amino acid changes in protein HBx.¹⁰ Natural Gene X mutations have been linked to the advancement of chronic liver disease owing to the decline of antiproliferative and apoptotic actions on infected hepatocytes, which contributes to carcinogenesis.^{11,12} Chronic HBV infection is known as the most common underlying etiology of hepatocellular carcinoma.¹³ Several mechanisms, including viral DNA integration,

the shortened form of HBsAg, and the HBx protein, have been postulated to connect HBV infection to HCC development.¹⁴ The HBx protein is known to operate wide cellular function, as a transcriptional activator of numerous cellular genes, to activate cell survival signaling pathways, to interact with oncogenic cellular proteins, and to produce epigenetic alterations. The HBx protein is also involved in the apoptotic process and has both pro and anti-apoptotic properties.¹⁵⁻¹⁷

The primary aim of this study was to investigate the mutation of the Gene X and its protein in asymptomatic CHB patients that infected with different genotypes and identifying the prevalence of 127, 130, and 131 amino acid HCC-related mutations in CHB clinical phase. We compared the Gene X sequence from our research sample to comparable GenBank sequences and published literature on clinically significant Gene X variants. We explore significance genetic variability of Gene X in CHB patient in our population.

METHOD

This research was a descriptive cross-sectional study that took 38 sera positive HBsAg who visited the the Red Cross Center (Padang, Indonesia). The inclusion criteria in this study was regular donor (at least 4 times in a year) that recorded over 1 year has persistent HBsAg. Exclusion criteria in this study was sample coinfectd with HCV or HIV and poor quality sample. The positive control that used in this study is Hepatitis B surface antigen (HBsAg)-positive serum that already confirmed by sequencing method (100%) prior the study. The study was conducted after obtaining approval from the Research Ethics Committee. HBV DNA Extraction: HBV DNA was

extracted using the Qiamp DNA Extraction Kit (Qiagen, Inc., Hilden, Germany) using procedures in accordance to the kit. Controls were treated as the same as sample. Precautions were followed to avoid cross contamination and appropriate negative and positive controls were included during DNA extraction and PCR amplification steps. The HBV Gene X was amplified using nested PCR. HBV Gene X region was amplified by a PCR protocol and used two set primers as follows: HBx1233F (5'- catgctggr (a/g) acctttgtg -3'), HBx1685R (5'- gcctcaaggtcggtcgtt -3'), HBx1545F (5'- ctcccc gtctgtgccttc -3'), HBx1940R (5'- cagaaggcaaaaaagaga gtaactc -3').¹⁸ The amplification was performed at 94°C for 30s, 50°C for 1 min, 72°C for 1 min with final elongation 72°C for 10 minutes.

Detection of PCR product with electrophoresis: PCR product was examined by electrophoresis using 1.5% agarose gel which indicated the expressed band, i.e 707 bp. The PCR product from the first round PCR was then used as DNA template for the second round PCR. In the second round PCR, Gene X was amplified into two fragments with size 488 bp and 422 bp. Analysis of gene X mutation: genotype and homology analysis with basic local alignment search tool (BLAST) from National Center of Biotechnology Information (NCBI) and hepatitis B virus database (HBVdb) were performed on this nucleotide sequences from this study and continued with comparing the sequence with international database according to the genotype results. The sequence result were analyzed with Geneious computer software to determine the mutation in the region. Phylogenetic tree analysis: The genotypes were confirmed based on the information contained within the references and by phylogenetic analysis. Multiple alignment were carried out using Geneious software.

RESULTS

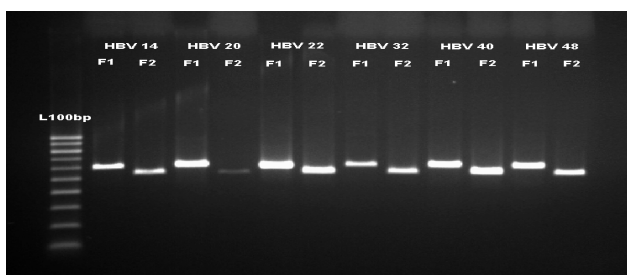


Figure 1. Electrophoregram result of Gene X_F1_F2 fragment for several samples (14,20,22,32,40, 48)

In this study, of the 38 HBV DNA samples that were positive by HBsAg sera, the entire Gene X region could be amplified from a total only 20 (52%) samples (Figure 1), suggesting the absence of this region in the rest of the 18 samples. Among the rest of the 18 samples in which full-length Gene X could not be amplified, our attempts to extract and amplify partial Gene X using published primers also failed repeatedly. The extracts were repeatedly found to be positive for PCR assays amplifying regions of Pre S, S, and C, ruling out the possibility of faulty DNA extraction in these samples. Two samples that were successfully amplified had poor sequencing quality, so the 18 remaining samples were analyzed further.

This study showed that all 18 samples had mutation in protein X. The result showed the presence of known HCC-related HBx mutants, i.e V5L (72%), I127T/S (44%), K130M/V131I (61%), G22S (11%) and R87W(5%). This analysis revealed that the triple mutation V5L/K130M/V131I was 55% and I127T/S/K130M/V131I was 55%. Analysis of the 76 retrieved genome sequences showed that patients were infected mainly by genotype C at 72,2% and followed by B at 27,8%. There is no recombinant detected. Presence of double mutant was more frequent in genotype C and all the triple mutation was found at the genotype C.

DISCUSSION

This study found the Gene X mutant A1762T/G1764A located in the overlapped BCP in the Gene X. These hotspot mutations are often found together. The nucleotide mutations resulted in overlapping amino acid changes, namely K130M and V131I. Double mutations in the nucleotide A1762T/G1764A (K130M +V131I) were the most common mutations found, as many as 61%.¹⁹ The mutation at BCP increase especially in the asymptomatic to chronic progression phase. According to a recent study, this mutation has a significant rising prevalence throughout the clinical phase of liver disease to HCC.²⁰⁻²² Patients with the combined BCP variant A1762T/G1764A were associated with an incidence of HCC.²³ The exact mechanism is unknown, but these mutations can accumulate during viral infection, resulting in genome instability and severe liver damage.²⁴⁻²⁷ This is related to suppression of pre-core messenger ribonucleic acid (mRNA), increased expression of hypoxia inducible factor 1 alpha (HIF-1 α) and increased transactivation when compared to wild-type. The overlapping of the HBV promoter core with the X gene coding sequence

results in a DNA mutation that will affect the regulatory function by suppressing p21 expression as a regulator and increasing pre genomic ribonucleic acid RNA (pgRNA) transcriptional expression, resulting in increased viral replication with uncontrolled cell proliferation. This is thought to have a greater impact on viral survival than the incidence with one mutation alone.^{19, 28-30}

The G1386C mutation results in simultaneous amino acid changes at codon 5, namely V5L located in microRNA binding region that reported tumor suppressor microRNA 15a/16 (miR-15a/16) induces B cell lymphoma 2 (Bcl2) and prevents apoptosis.^{31,32} The V5L as the negative domain of the HBx regulator (which functions as transactivation) reported significantly found in an HBeAg-negative serostatus. This mutation also increase the activity of major nuclear factor kappa B (NF-κB), thus become important and specific mutation in the incidence of HCC risk by 5.34 times. Most of samples with V5M in this study also had double mutations in K130M+V131L which was 55%. When compared to only double mutations, triple mutations in chronic HBV are associated with an increased risk of tumorigenesis. There is no increase in viral replication activity with this triple mutation, whether they had the double mutation or only V5M mutation, indicating that this triple mutation is not directly related to viral protein expression.^{19,33,34}

A mutation at the T1753V point results in a change in the I127T/S amino acid that affects the binding affinity of HBx to Bcl2, thereby inducing apoptosis, increasing risk of HCC and liver cirrhosis.³⁵ Several other studies have shown that the triple mutation in I127T/S is associated with the K130M+V131I mutation. The mutation at codon 127 reported will appear later as a result of alleged polar mutation of K130M+V131I.³⁶ This study showed that 44% of the samples were found to have triple mutations. The mutation at codon 120-140 affect the functional domain associated with nuclear transactivation and signal transduction, therefore this mutation associated with hepatocarcinogenesis modulation.³⁷⁻³⁹ A recent study also observed this mutation has significant existence to accelerate clinical phase of liver disease to HCC.²⁰

This study revealed that the most HBV strains isolated belonged to genotype C and B. Despite the fact that the HBV genotype is commonly specified using the S gene, phylogenetic trees constructed for the Gene X appear to be reliable for classifying the HBV genotype. According to this study, HBV genotype C was found

to be more prevalent than genotype B. Another study from Sulawesi Island discovered a higher percentage of genotype C.¹⁸ In western Indonesia, genotype B predominated, while genotype C predominated in eastern areas.^{4,40} An accordance to previous research, genotype C is the most abundant in Minangkabau ethnic group and is associated with social interactions among the ethnic.⁴¹ Recent study in Pekanbaru revealed that the pattern domination of genotype C.⁴² These findings support the idea that geography and ethnicity influence the distribution of HBV genotypes.

Genotype has important influence in HBeAg and HBcAg seroconversion, viremia levels, mutations and disease progression.⁴³ In contrast to genotype C, genotype B as spontaneous seroconversion at a younger age and a slower progression to liver cirrhosis. Genotype C is thought to have a more severe disease course because it has been linked to liver fibrosis and delayed HBeAg seroconversion due to high viral reactivation. This is due to specific mutations in the BCP promoter and pre-Core region which are also linked to an increased risk of HCC.^{41,44-46} These genotypes also cause differences in HBV therapy response and resistance. Genotype B responded better to interferon (IFN)- and Lamivudine therapy than genotype C.⁴⁷ Patients infected with genotype C were more likely to respond to pegylated interferon (PEG-IFN) than standard IFN.⁴⁸⁻⁵⁰

CONCLUSION

This study found all the asymptomatic CHB samples have already nucleotide mutation. This study implies that molecular progression between the virus and the host is could started before liver disease such as cirrhosis and HCC occurred

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