MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.10, No.1, March 2016, p 9-14

Available online at
http://jurnal.permi.or.id/index.php/mioline
DOI: 10.5454/mi.10.1.2

Detection of Hepatitis B Virus X Gene Mutation from Local Clinical Samples

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Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family and it infects hepatocytes, which is the most common cell in liver. HBV infection is distinguished into acute and chronic infection based on the duration of infection. Chronic infection of HBV exists more than six months and it can develop into liver cirrhosis and hepatocellular carcinoma (HCC). The development of chronic HBV infection is affected by viral particle load, genotype and subgenotype, as well as its association with HBV X protein (HBx). Several mutations of the HBx gene are known to be associated with HCC. The aim of this study is to determine the genotype and subgenotype based on HBx gene, and to detect its mutation from 44 local clinical samples. The HBx gene was amplified using nested PCR, which produced two fragments with size of 469 and 395 bp. The obtained HBx gene sequences were aligned with HBx gene sequences from database to determine the genotype, subgenotype, and amino acid substitution. The analysis showed that patients were infected mainly by HBV subgenotype B3, which are common in Indonesia. In this study, we found the presence of known HCC-related HBx mutant, i.e. V5L, A47T, I127T and K130M/V131I, as well as new HBx mutant, i.e. G22S and A85T. The presence of HBx T118N mutant was detected at the highest percentage and occurred from samples with high HBV DNA titer. Detection of HBx gene mutations might be necessary to predict the manifestation of liver disease, as well as development of HCC.

Key words: hepatitis B virus, Indonesia, X gene mutation

Virus Hepatitis B (VHB) tergolong dalam famili *Hepadnaviridae* dan dapat menginfeksi sel hepatosit manusia. Infeksi VHB dapat terjadi secara akut dan kronis berdasarkan lamanya infeksi. Infeksi kronis terjadi selama lebih dari enam bulan dan dapat berkembang menjadi sirosis hati dan karsinoma hati. Perkembangan infeksi VHB kronis menjadi sirosis dan karsinoma hati dipengaruhi oleh jumah virus yang menginfeksi, genotipe dan subgenotipe VHB, dan hubungannya dengan fungsi protein X VHB (HBx). Mutasi pada gen pengkode HBx diketahui berasosiasi dengan status karsinoma hati. Tujuan penelitian ini adalah menentukan genotipe dan subgenotipe VHB dan mendeteksi mutasi gen pengkode HBx dari 44 sampel lokal. Gen pengkode HBx diamplifikasi menggunakan metode *nested* PCR yang menghasilkan 2 produk PCR berukuran 469 dan 395 bp. Urutan nukleotida gen pengkode HBx disejajarkan dengan urutan gen pengkode HBx di database untuk penentuan genotipe, subgenotipe, dan substitusi asam amino HBx. Hasil analisis menunjukkan keberadaan genotipe B dan subgenotipe B3 terdapat secara dominan. Hasil analisis urutan gen pengkode HBx menunjukkan substitusi asam amino HBx yang telah diketahui berasosiasi dengan karsinoma hati, yaitu V5L, A47T, I127T dan K130M/V131I. Hasil analisis juga menunjukkan substitusi asam amino baru, yaitu G22S and A85T. Keberadaan HBx T118N mutan juga dideteksi pada penelitian ini dengan presentase tertinggi dan terdeteksi dari sampel dengan titer DNA VHB tinggi. Deteksi mutasi pada gen pengkode HBx dapat digunakan lebih lanjut sebagai prediksi manifestasi klinik infeksi hati oleh VHB dan kaitannya dengan perkembangan karsinoma hati.

Kata kunci: Indonesia, mutasi gen X, virus hepatitis B

Liver cancer is one of the most common cancers worldwide with high mortality rate (Ferlay *et al.* 2013) and it occurs mainly in the form of hepatocellular carcinoma (HCC). Approximately 80% of the HCC cases are due to chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (El-Serag 2012). HBV belongs to the *Hepadnaviridae* family of virus and is the causative agent of acute and chronic liver diseases. While the acute HBV infection permits full recovery, the chronic HBV infection lasts for more than 6 months, defined by persistence of HBsAg (Chang 2007). Increased risk of HCC development is observed in patients having chronic HBV infection and it can occur in absence of liver cirrhosis (Chemin and Zoulim 2009; El-Serag 2012).

HBV is classified into ten genotypes, i.e. genotype A – H, with genotype B and C predominantly exist in Indonesia (Sunbul 2014). The HBV genotype C is known to cause severe liver disease, cirrhosis, and HCC as compared to the HBV genotype B. However, HBV genotype B correlates with HCC incidence without cirrhosis (El-Serag 2012). Several mechanisms have been proposed to link HBV infection

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and HCC development, i.e. viral DNA integration, the truncated form of HBsAg and the X protein of HBV (HBx) (Di Bisceglie 2009). Integration of viral DNA to the host genome appears to happen at random and it was found in 80% of HBV infected patient with HCC. The mechanism of truncated HBsAg in causing HCC remains unclear, whereas the HBx protein may contribute to HCC development in many ways. The HBx protein is known to act as a transcriptional activator of many cellular gene, activate cell survival signaling pathway, interact with cellular protein which has oncogenic potential and cause epigenetic change (Di Bisceglie 2009; Kew 2011; Shlomai *et al.* 2014). The HBx protein can modulate signaling transduction pathways, i.e. Ras, Raf, MAPK, NF-ĸB and Jak-STAT, which then leads to stimulation of hepatocyte proliferation. The HBx protein also plays a role in apoptosis process and has pro- and anti-apoptotic effects (Kew 2011).

Several HBx mutant proteins have been reported to be correlated with HCC incidence, i.e. V5M/L, P38S, H94Y, I127T/N, and K130M/V131I (Cho *et al.* 2011; Jang *et al.* 2012; Kim 2014). It has been reported that the K130M/V131I HBx mutation was present in 51% of HCC tissue (Liu *et al.* 2014), whereas the T118N mutant was present in 50% and 30% of chronic hepatitis (CH) and HCC samples, respectively (Utama *et al.* 2009). Another study also reported the existence of T118N mutant in one out of ten samples from Manado (Fatimawali *et al.* 2014). The C-terminally truncated HBx was observed in 30-40% HCC patients, more frequently in tumor samples (Liu *et al.* 2008; 2014; Sze *et al.* 2013). A number of studies have also explored the contribution of HBx mutants to development of hepatocarcinogenesis. The K130M/V131I HBx mutant protein was shown to upregulate HIF-1α expression and transactivation activity as compared to the wild-type HBx (Lee *et al.* 2014; Liu *et al.* 2014), as well as differentially influencing cell proliferation (Lin *et al.* 2005; Iyer and Groopman 2011).

In this study, we analyzed the HBx gene sequences from 44 local clinical samples. We also determined the genotype, subgenotype and the HBx gene mutation of the samples. The HBx gene was amplified using nested PCR and the sequence of HBx gene was analyzed in comparison to the existing sequence in the database. The amino acid substitution of HBx protein was compared to the known HBx mutant, especially the HCC-related HBx mutant, as well as determination of new HBx mutant.

MATERIALS AND METHODS

PCR and DNA Sequencing. HBV DNA samples were isolated at Pramita Diagnostic Laboratory Jakarta, Indonesia, including the determination of HBV DNA titer in the samples. The HBx gene was amplified using nested PCR. Primer pairs were designed according to the sequence of HBV genome in the GenBank database. Primers used for the first round of PCR were designed to recognize the region outside the HBx gene at nucleotide 1233 and 1940 of HBV genome. For the second round of PCR, additional primers were designed at position 1685 and 1545 of the HBV genome. The primers used for the PCR reactions are as follows: HBVF1_1233_for (5'-CATGCGTGG RCA/GCCTTGTG-3'), HBVF1_1685_rev (5'-GCCTCAAGGTCGGTCGTT-3'), HBVF2_1545_for (5'-CTCCCCGTCTGTG CCTTC-3') and HBVF2_1940_rev (5'-CAGAAGGCAAAAAA GAGACTAACTC-3'). The PCR mixture for the first round of PCR contained 100 ng of HBV DNA, 0.4 µM of each primer HBVF1_1233_for and HBVF2_ 1940_rev, 1X DreamTaq Green Master Mix (Thermo Scientific) and PCR-grade water. The amplification was performed at 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, with final elongation at 72 °C for 10 min. At the second round of PCR, the amplification of HBx fragment 1 (HBx_F1) used HBVF1_1233_for and HBVF1_1685_rev primer pair, while the the amplification of HBx fragment 2 (HBx_F2) used HBVF2_1545_for and HBVF2_1940_rev primer pair. The second round of PCR used 1 µL of 1/25 diluted PCR product from the first round of PCR as DNA template. The PCR product from the second round of PCR was analyzed using 1.5% agarose gel electrophoresis. Positive results were shown by existence of 469 bp of HBx_F1 and 395 bp of HBx_F2. Specific DNA bands of HBx_F1 and HBx_F2 from 44 samples were then purified using Gel or PCR DNA Fragments Extraction Kit (Geneaid). The samples were subjected to DNA sequencing at Macrogen, Korea. Confirmation of the nucleotide changes were performed by re-sequencing of the PCR product.

Sequence Analyses. Raw sequences from DNA sequencing was confirmed using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the sequences of HBx_F1 and HBx_F2 were then assembled. Determination of genotype and subgenotype was based on the similarity to the sequences deposited in the NCBI database. Analysis of

amino acid substitution was performed by translating the DNA sequence using ExPASy-Translate Tool (http://web.expasy.org/translate/) and then aligning the protein sequences with several HBx protein sequences from the database using MultAlin program (http://multalin.toulouse.inra.fr/multalin/).

RESULTS

To determine the nucleotide sequence, the HBx gene was amplified using nested PCR. The first round of PCR yielded 707 bp of PCR product which was undetectable in agarose gel electrophoresis. The PCR product from the first round PCR was then used as DNA template for the second round of PCR. In the second round of PCR, HBx gene was amplified into 2 fragments, HBx_F1 and HBx_F2. The result from HBx_F1 amplification from several clinical samples showed band with experimental size of 463 bp, indicating presence of HBx_F1 with theoretical size of 469 bp (Fig 1). Meanwhile, the amplification of HBx_F2 showed band with size of 403 bp, which was in accordance to HBx_F2 theoretical size of 395 bp (Fig 2).

To analyze the nucleotide sequence of HBx gene, purified PCR product was analyzed by direct sequencing. The DNA sequences were then assembled and the complete or partial sequences of HBx gene was analyzed (GenBank NCBI accession number KX429616-59). Determination of the genotype and subgenotype of HBV was performed by BLAST analysis based on the similarity of the HBx gene to the NCBI database. The result showed that genotype B, C and D were detected in 77.3%, 20.5% and 2.3%, respectively. Analysis of subgenotype showed that B2, B3, C1 and C2 subgenotypes were detected in 18.2%, 59.1%, 15.9% and 4.5%, respectively (Table 1). From the analyzed samples, genotype B and subgenotype B3 occured at the highest percentage.

To analyze the HBx amino acid substitution, DNA sequence results were translated and aligned with HBx protein sequences from the database with same genotype or subgenotype. Some known HCC-related HBx gene mutation as well as new HBx mutation which occured in two or more samples were listed in Table 2. The result showed the presence of known HCC-related HBx mutant, i.e. V5L (2.3%), A47T (6.8%), I127T (4.5%) and K130M/V131I (13.6%) (Table 2). Presence of HBx R87W was observed at 4.5% (Table 2) and this mutant has been observed previously from HBV genotype F in chronic carrier patients (León *et al.* 2005). We also observed the presence of HBx V45L, G22S and A85T mutants in 11.4%, 9.1% and 4.5%, respectively. The HBx V45L was detected from samples with genotype B2, which was in accordance to the previous report (Fatimawali *et al.* 2014). The presence of HBx G22S and A85T mutants was never been reported before.

In this study, the HBx T118N mutant occurred at highest percentage (15.9%), while the HCC-related

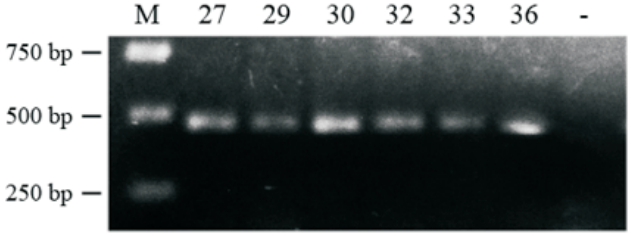

Fig 1 Electrophoregram result of HBx_F1 PCR product. Amplification of HBx gene was performed by nested PCR from several clinical samples (27, 29, 30, 32, 33) to obtain HBx_F1 fragment. The PCR product was analyzed by 1.5% agarose gel electrophoresis. (M) : 1kb DNA Marker, (-): PCR negative control.

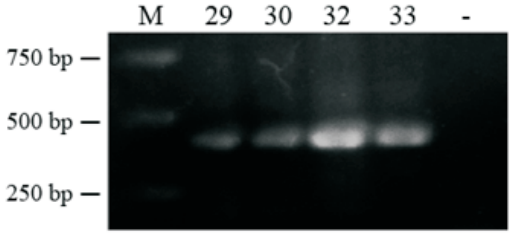

Fig 2 Electrophoregram result of HBx_F2 PCR product. Amplification of HBx gene was performed by nested PCR from several clinical samples (29, 30, 32, 33) to obtain HBx_F2 fragment. The PCR product was analyzed by 1.5% agarose gel electrophoresis. (M) : 1kb DNA Marker, (-): PCR negative control.

K130M/V131I mutant occurred at 13.6% (Table 2). To date, the existence of HBx T118N was observed only from study using samples collected in Indonesia (Utama *et al.* 2009; Fatimawali *et al.* 2014) and its direct correlation to HCC incidence was not investigated so far. The presence of HBx T118N mutant was observed from samples having high HBV DNA titer ($\geqslant 10^6$IU mL^{-1}), similarly to the known HCC-related HBx K130M/V131I mutant (Table 2).

DISCUSSION

Detection of HBV genotypes, subgenotypes and HBx gene mutations have been studied intensively to find association with HCC development. Therefore, the studies may contribute in understanding clinical implication of diverse HBV genotypes and HBx gene mutation, as well as in finding better management of HBV infected patients. A meta-analyses study performed based on published data from 1950 to 2012 showed that HBV genotype C correlates with higher risk of HCC as compared to other HBV genotypes. Association of HBV genotype B and C to HCC was found at 12% and 25%, respectively (Wong *et al.* 2013). The HBx gene mutation may as well associates with HCC incidence, escpecially mutation that occurs in the HBx transactivation domain, protein binding site and immune epitopes (Li *et al.* 2015).

In this study, amplification of the HBx gene was performed using nested PCR which resulted 2 HBx fragments in the final PCR product. Using this approach, amplification and HBx gene sequencing was successful for all 44 samples and for samples with HBV DNA titer as low as 10^6 IU mL^{-1}. Previous attempt using conventional PCR showed successful amplification only for 12 out of 20 samples and successful nucleotide sequence analysis only from 5 out of 12 amplified samples. The amplification of HBx gene using conventional PCR was also only successful for samples with HBV DNA titer of 10^8 IU mL^{-1} (Gunawan 2012).

Based on this research, HBV genotype B was found at higher percentage in comparison to genotype C and D. This is in accordance to previous report that genotype B is the most abundant HBV genotype found in Indonesia, followed by genotype C (Utama *et al.* 2009; Sunbul 2014). It is previously shown that genotype B is found more abundantly in West area of

Table 1 HBV genotype and subgenotype based on HBx sequence

Genotype	Subgenotype	n (%)
B	B2	8 (18.2)
	B3	26 (59.1)
C	C1	7 (15.9)
	C2	2 (4.5)
D		1 (2.3)

Table 2 Frequencies of some HBx mutations

Amino acid substitution	n (%)	Sample (HBV DNA titer in IU mL^{-1})
V5L*	1 (2.3)	43 (10^8)
G22S	4 (9.1)	18 (10^8), 26 (10^7), 28 (10^7), 34 (10^8)
V45L	5 (11.4)	3 (10^7), 17 (10^6), 19 (10^6), 22 (10^5), 25 (10^5)
A47T*	3 (6.8)	26 (10^7), 29 (10^8), 31 (10^7)
A85T	2 (4.5)	41 (10^8), 42 (10^7)
R87W	2 (4.5)	5 (10^7), 15 (10^6)
T118N	7 (15.9)	1 (10^8), 7 (10^6), 9 (10^7), 12 (10^6), 29 (10^8), 33 (10^8), 42 (10^7)
I127T*	2 (4.5)	7 (10^6), 43 (10^8)
K130M/V131I*	6 (13.6)	2 (10^6), 4 (10^7), 7 (10^6), 15 (10^6), 40 (10^7), 43 (10^8)

*known HCC-related HBx mutation

Indonesia while genotype C is predominant in East Indonesia (Thedja *et al.* 2011). The samples analyzed in this study were obtained predominantly from Sumatra and Java islands, thus more genotype B was shown. Meanwhile, another study performed with samples from Sulawesi island showed that genotype C was found at higher percentage (Fatimawali *et al.* 2014). This study also showed that subgenotype B3 was the predominant HBV subgenotype. This is in accordance to the previous report that subgenotype B3 are the most abundant HBV subgenotype found in Sumatra and Java islands (Thedja *et al.* 2011).

In this study, analysis of HBx gene sequence showed the existence of known HCC-related mutants, such as V5L, A47T, I127T and K130M/V131I, with HBx K130M/V131I occurred at higher percentage as compared to the other HCC-related mutants. Meanwhile, existence of HBx mutant T118N was detected at the highest percentage, 15.9%. In previous report, HBx T118N was found in 50% of CH patients and in 31% of HCC patients. Meanwhile, the HBx K130M/V131I was found in 37% of HCC patients (Utama *et al.* 2009). The percentage of HBx K130M/V131I and HBx T118N detected in this study were rather low because it was detected from all HBV-infected patients and the clinical status of the patients was unknown. To date, there were no reports investigating the correlation of HBx T118N mutant to development of HCC, while there have been many reports studying mechanism on how HBx K130M/V131I contributes to HCC *in vitro*. As the HBx T118N was found merely in samples with high HBV DNA titer and detected as abundant as HBx K130M/V131I in Indonesia, it might be necessary to study its correlation to HCC development. In conclusion, our study highlight the existence of known HCC-related HBx mutants as well as other previously uncharacterized HBx mutants. Our observation of HBx T118N mutant strengthens the evidence of HBx mutant specifically occurred in Indonesia. This study also pinpoints possible correlation of HBx T118N in HCC development, which needs to be investigated further.

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