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Association Between HBx Variations and Development of Severe Liver Disease Among Indonesian Patients

WAHYU ARISTYANING PUTRI[1], YOSHIHIKO YANO[1,2*], LAURA NAVIKA YAMANI[3], MARIA INGE LUSIDA[3], SOETJIPTO[3], YUJIAO LIANG[4], YAN MARDIAN[4], WIDYA WASITYASTUTI[5],and YOSHITAKE HAYASHI[4]

[1]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan;
[2]Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan;
[3]Indonesia–Japan Collaborative Research Centre for Emerging and Re-emerging Infectious Disease, Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia;
[4]Division of Molecular Medicine & Medical Genetics, Department of Pathology, Kobe University Graduate School of Medicine, Kobe, Japan
[5]Department of Physiology Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
**Corresponding author*

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Multi-site mutations in the hepatitis B virus (HBV) X gene are often found in patients with advanced liver diseases such as liver cirrhosis and hepatocellular carcinoma. It has been reported that modifications in the X protein play crucial roles in the development of HBV-related severe liver disease. However, the prevalence of genetic variations in Indonesian strains has not been systematically assessed. In this study, we sought to investigate the profile of nonsynonymous mutations in the X gene. Overall, 114 Indonesian HBV strains, including 12 in-house samples, were retrieved from GenBank. The mutation frequency in the X gene was compared among strains obtained from patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The mutation frequencies of the domain and basal core promoter regions were significantly greater in advanced liver diseases compared with chronic hepatitis. In addition, the double mutation K130M/V131I and the triple mutation N88V/K130M/V131I were associated with a 2.5 times higher risk of advanced liver disease. However, the roles of two novel X gene mutations (A12S/T and L16F/P) on hepatocarcinogenesis are unclear relative to wild-type X gene. In conclusion, the development of multi-site mutations in the X gene may represent a strategy by which HBV can escape immune surveillance and thus contribute to hepatocarcinogenesis, even though the biological roles of some variants remain unclear.

INTRODUCTION

Hepatitis B virus (HBV) X protein is a 154 amino-acid-long protein that acts as a pleiotropic transactivator (1). It is a multifunctional protein that activates transcription by interacting with nuclear transcription factors and modulates cytoplasmic signal transduction pathways (2, 3). It has been suggested that its transcriptional regulatory activities and activation of gene promotors are dependent on the N-terminal half of the full-length 17 kDa protein (4). X protein is encoded by the X gene, which contains a region that overlaps several structural and functional sequences in the viral genome (5). Due to the overlapping coding and regulatory elements in the X gene, DNA mutations and deletions can affect gene and transcriptional regulatory functions (1). Mutations may accumulate during HBV infection and cause DNA damage, resulting in genomic instability and eventually leading to the development of severe liver disease (6-8). There are two dominant types of X gene mutations in chronic disease: type I mutations, which are single nucleotide mutations occurring at multiple sites; and type II mutations, which are C-terminal truncations (1). A recent study examining the pathogenesis of X gene-induced hepatocellular carcinoma (HCC) and other etiological tumor types suggested that several factors contributed to HCC pathogenesis, including DNA repair, X gene methylation, non-coding RNA, and X gene mutations (9-11). Multi-site variations in the X gene are often found in patients with advanced liver disease although the pathological role of each point-mutation needs further investigation (10, 12, 13). Variations in the X gene in Indonesian strains have not been systematically assessed. Therefore, this study sought to investigate the association between nonsynonymous X gene mutations in Indonesian HBV strains and development of advanced liver disease.

Phone: +81-78-382-6305 Fax: + 81-78-382-6309 E-mail: yanoyo@med.kobe-u.ac.jp

METHODS

Retrieval of HBV genomes from GenBank

To identify naturally occurring mutations in the X gene among Indonesian HBV strains, we retrieved 114 HBV genome and protein sequences, including 12 in-house samples, by searching GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) with the following keywords: hepatitis B virus, X gene, Indonesia. All sequence data used in this study are available under an open source license (NCBI database) and should have clinical information. The full-length sequences of strains belonging to genotypes B and C were included in this study. Recombinant strains beyond genotypes B or C were excluded from the analyses. The study protocol was approved by the Ethics Committees at Kobe University, Japan, and the Institute of Tropical Disease, Airlangga University, Indonesia.

Phylogenetic tree analysis

The genotypes were confirmed based on the information contained within the references and by phylogenetic analysis. Multiple alignments were carried out using Clustal W software (https://www.genome.jp/tools-bin/clustalw). A phylogenetic tree was constructed using the minimum evolution (ME) method based on Kimura two-parameter distance estimation. To confirm the reliability of the phylogenetic tree topologies, bootstrap reconstruction was carried out 1,000 times. Analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.2 (http://megasoftware.net/).

Analysis of amino acid substitutions

The protein sequences retrieved from GenBank were confirmed using All in One Sequence Analyzer software (http://www-personal.umich.edu/~ino/blast.html). The amino acid substitutions caused by mutations in the X gene were analyzed by searching for sequence similarities using FASTA (http://fasta.sbioch.virginia.edu/fasta_www2/fasta_list2.shtml) among the sequences obtained from patients with chronic hepatitis B (chronic hepatitis group) or patients with HBV-related advanced liver diseases, including liver cirrhosis and HCC (advanced liver disease group).

Calculation of the mutation frequency index

The mutation frequency index (MFI) represents the number of mutations relative to the length of the gene. The MFI was calculated for the coding and regulatory domains of the X gene. The total number of amino acid mutations was calculated individually for these peptides in all of the samples within each diagnostic group (14). The MFI was then calculated using the following formula:

$$MFI = [x/(y \times z)] \times 100,$$

where x = total number of mutations observed in the amino acid sequences, y = total number of samples in the group, and z = length of the amino acid sequence.

Statistical analysis

All statistical analyses were performed using SPSS software version 22.0 (IBM, Armonk, NY, USA). Statistical significance was determined using parametric *t* tests and *P* values of <0.05 were considered statistically significant.

RESULTS

Summary of the genome sequences

Analysis of the 102 retrieved genome sequences and 12 in-house sequences showed that the dominant HBV genotypes in Indonesia were genotypes B and C (52.6% [60/114] and 47.4% [54/114], respectively) (phylogenetic tree not shown). Table I summarizes the accession numbers, clinical characteristics and publication history for all sequences. The clinical profile of each strain was also analyzed. Among the 114 samples, 38 were from patients with chronic hepatitis (33.3%), 50 from patients with liver cirrhosis (43.9%), and 26 from patients with HCC (22.8%).

Table I. Summary of the genome sequences

No of sequences	Gene accession number	Protein accession number	Genotype	Clinical status	Location	Reference
2	JQ429081, JQ429080	AFP89269, AFP89265	B	HCC	Jakarta/ Mataram/ Makasar/ Padang	(15)
1	JQ429078	AFP89257	C	CH		
2	JQ429079, JQ429082	AFP89261, AFP89273	B	LC		
23	JX196403, JX196407, JX196408, JX196416, JX196417, JX196419, JX196420, JX196422, JX196424, JX196426, JX196431, JX196441, JX196443, JX196444, JX196445, JX196486, JX196446, JX196453, JX196457, JX196458, JX196461, JX196464, JX196484	AFQ94843, AFQ94847, AFQ94848, AFQ94856, AFQ94857, AFQ94859, AFQ94860, AFQ94862, AFQ94864, AFQ94866, AFQ94871, AFQ94881, AFQ94883, AFQ94884, AFQ94885, AFQ94926, AFQ94886, AFQ94893, AFQ94897, AFQ94898, AFQ94901, AFQ94904, AFQ94924	B	LC	Padang	(16)
13	JX196412, JX196421, JX196428, JX196432, JX196434, JX196435, JX196436, JX196439, JX196451, JX196460, JX196465, JX196466, JX196474	AFQ94852, AFQ94861, AFQ94868, AFQ94872, AFQ94874, AFQ94875, AFQ94876, AFQ94879, AFQ94891, AFQ94900, AFQ94905, AFQ94906, AFQ94914	B	HCC		
20	JX196427, JX196414, JX196415, JX196425, JX196430, JX196438, JX196440, JX196442, JX196448, JX196449, JX196450, JX196454, JX196455, JX196459, JX196462, JX196463, JX196467, JX196469, JX196470, JX196473	AFQ94867, AFQ94854, AFQ94855, AFQ94865, AFQ94870, AFQ94878, AFQ94880, AFQ94882, AFQ94888, AFQ94889, AFQ94890, AFQ94894, AFQ94895, AFQ94899, AFQ94902, AFQ94903, AFQ94907, AFQ94909, AFQ94910, AFQ94913	B	CH		
18	JX196404, JX196409, JX196411, JX196418, JX196487, JX196489, JX196490, JX196492, JX196494, JX196495, JX196497, JX196452, JX196456, JX196478, JX196480, JX196481, JX196483, JX196485,	AFQ94844, AFQ94849, AFQ94851, AFQ94858, AFQ94927, AFQ94929, AFQ94930, AFQ94932, AFQ94934, AFQ94935, AFQ94937, AFQ94892, AFQ94896, AFQ94918, AFQ94920, AFQ94921, AFQ94923, AFQ94925,	C	LC		
10	JX196402, JX196406, JX196423, JX196437, JX196468, JX196475, JX196491, JX196493, JX196496, JX196498	AFQ94842, AFQ94846, AFQ94863, AFQ94877, AFQ94908, AFQ94915, AFQ94931, AFQ94933, AFQ94936, AFQ94938	C	HCC		
13	JX196400, JX196405, JX196410, JX196429, JX196433, JX196447, JX196471, JX196472, JX196476, JX196477, JX196479, JX196482, JX196488	AFQ94840, AFQ94845, AFQ94850, AFQ94869, AFQ94873, AFQ94887, AFQ94911, AFQ94912, AFQ94916, AFQ94917, AFQ94919, AFQ94922, AFQ94928	C	CH		
4	LC349872, LC349873, LC349875, LC349879	BBC20835, BBC20836, BBC20838, BBC20842	B	CH	Surabaya	Unpublished
7	LC349868, LC349869, LC349871, LC349874, LC349876, LC349877, LC349878	BBC20831, BBC20832, BBC20834, BBC20837, BBC20839, BBC20840, BBC20841	B	LC		
1	LC349870	BBC20833	B	HCC		

HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis

Profile of X region mutations

Analysis of the variations in the X region indicated that mutations were more frequent in the B cell epitope, Boxα and basal core promoter (BCP) regions (Figure.1). Statistical analysis showed that the frequencies of mutations in BCP (P = 0.019) and the domain (P = 0.007) were significantly greater in the liver cirrhosis group than in the chronic liver disease group (Table II). The domain is the transcriptional and regulatory region in the X gene, and the inter-domain is the region between two domains (17). The mutation frequency index in the domain region was also significantly greater in HCC than in chronic liver disease (P = 0.043) (Table II). Two mutations were significantly more frequent in HCC than in chronic liver disease: S43P (P = 0.032) and N88V (P < 0.001). Four mutations were significantly more frequent in liver cirrhosis than in chronic liver disease: S43P (P = 0.016), N88V (P < 0.001), K130M/X (P = 0.043), and V131I (P = 0.043) (Table II).

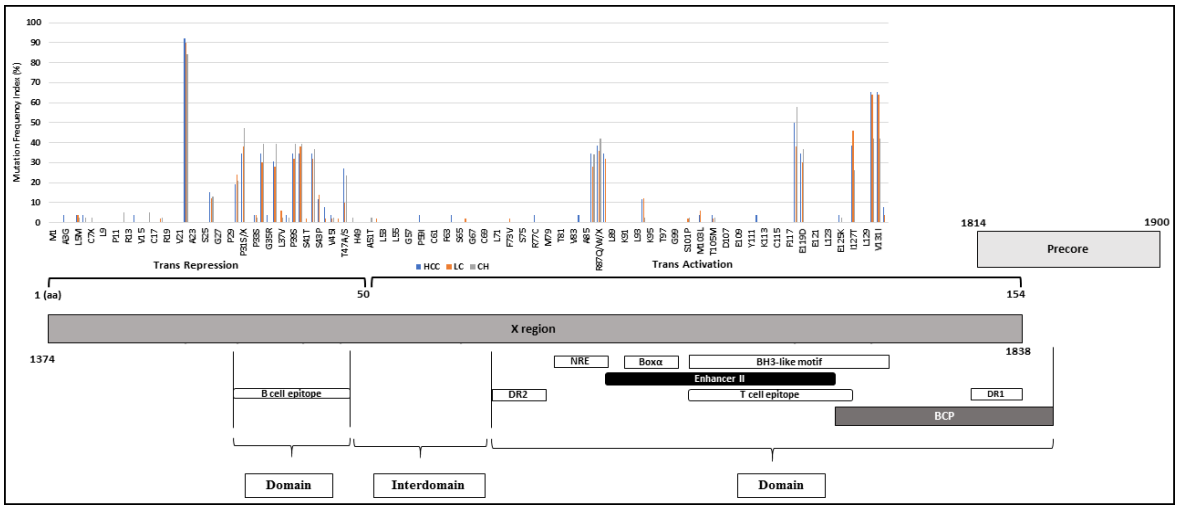

Figure 1. Location of mutations in the X region. The domain is the transcriptional and regulatory region in the X gene, and the interdomain is the region between two domains. The majority of mutations were located in the B cell epitope, Box alfa, and BCP regions. DR2, Direct Repeat 2; NRE, Negative Regulatory Element; Boxα, Box alfa; BCP, Basal Core Promoter; DR1, Direct Repeat 1

Table II. Comparison of mutation sites among patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma

Mutation	Mutation frequency index (%) (mean ± SD)			*P* value		
	CH	LC	HCC	CH vs. LC	CH vs. HCC	LC vs. HCC
S43V	0.000 ± 0.000	14.00 ± 35.10	11.50 ± 32.60	0.016*	0.032*	0.762
N88V	0.000 ± 0.000	32.00 ± 47.10	34.60 ± 48.50	<0.001**	<0.001**	0.823
K130M	42.10 ± 50.00	64.00 ± 48.50	65.40 ± 48.50	0.043*	0.068	0.906
V131I	42.10 ± 50.00	64.00 ± 48.50	65.40 ± 48.50	0.043*	0.068	0.906
Trans repression	8.368 ± 6.784	7.440 ± 6.021	8.154 ± 7.309	0.507	0.906	0.671
Trans activation	2.807 ± 2.666	3.524 ± 3.013	3.919 ± 3.368	0.241	0.166	0.617
B cell epitope	15.000 ± 16.886	13.200 ± 15.077	14.231 ± 17.245	0.606	0.860	0.798
T cell epitope	10.307 ± 8.758	9.500 ± 9.673	10.577 ± 10.424	0.683	0.914	0.663
BCP	4.526 ± 5.192	7.120 ± 4.801	7.231 ± 5.659	0.019*	0.058	0.933
Enhancer II	2.851 ± 2.701	3.389 ± 4.358	3.953 ± 4.456	0.505	0.223	0.600
Direct Repeat 2	0.000 ± 0.000	0.400 ± 2.828	0.769 ± 3.922	0.322	0.230	0.673
Enhancer binding protein	0.526 ± 3.244	2.400 ± 6.565	2.308 ± 6.516	0.110	0.153	0.954
Negative Regulatory Element	9.540 ± 11.781	8.000 ± 11.226	9.615 ± 11.889	0.537	0.980	0.570
Box alfa	0.376 ± 2.317	1.714 ± 4.689	1.648 ± 4.654	0.110	0.153	0.954
BH3-like motif	12.229 ± 10.888	14.471 ± 10.906	15.611 ± 12.220	0.342	0.261	0.691
Domain	2.451 ± 2.959	4.417 ± 3.712	4.440 ± 4.185	0.007*	0.043*	0.982
Inter-domain	0.800 ± 0.744	0.624 ± 0.797	0.769 ± 0.861	0.290	0.883	0.478
Genotype C	0.368 ± 0.489	0.360 ± 0.485	0.385 ± 0.496	0.936	0.898	0.837
Genotype B	0.632 ± 0.489	0.640 ± 0.485	0.615 ± 0.496	0.936	0.898	0.837

*P < 0.05 and **P < 0.01 denote significant between-group differences CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Association between X gene mutations and development of advanced liver disease

The MFI in the regulatory elements of the X gene was analyzed and compared between the chronic hepatitis and advanced liver disease groups. As shown in Figure. 2, the MFI in the BCP was significantly different between the chronic hepatitis and advanced liver disease group. In addition, the mutations N88V (P < 0.001), K130M (P = 0.026), and V131I (P = 0.026) were more frequent in advanced liver disease than in chronic hepatitis. Although the mutations A12S/T (P = 0.044) and L16F/P (P = 0.044) were less frequent in advanced liver disease (Table III), the roles of these two novel X gene mutations on hepatocarcinogenesis are unclear relative to wild-type X gene. Based on odds ratios, a patient with the mutations K130M (P = 0.025) or V131I (P = 0.025) had a 2.5 times higher risk of advanced liver disease than a patient without these mutations (Table V). Patients with the double mutation K130M/V131I (P = 0.049) and the triple mutation N88V/K130M/V131I (P < 0.001) had 2.2 times higher risk of advanced liver disease than patients without the double or triple mutation (P = 0.048 and P = 0.048, respectively) (Table V).

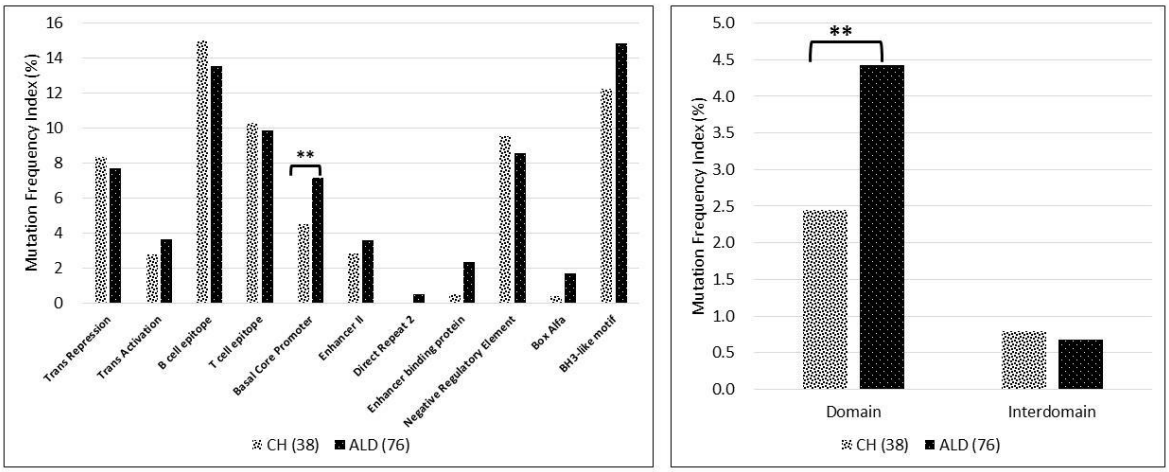

Figure 2. Comparison of the mutation frequency index (MFI) within regions of the X gene between patients with chronic hepatitis and advanced liver disease. (A) MFIs of specific open reading frames in the X gene. (B) MFIs in the domain and interdomain regions. *P < 0.05 and **P < 0.01 denote significant differences between groups. CH, chronic hepatitis; ALD, advanced liver disease

Association between X gene mutations and development of HCC

To determine which X gene mutations were associated with development of HCC, the strains were divided into HCC and non-HCC groups. This analysis revealed that the triple mutation N88V/K130M/V131I was significantly more frequent in the HCC group ($P = 0.024$)(Table IV) and increase the risk for HCC by 3.054 fold (95% CI = 1.124 to 8.300, $P = 0.029$)(Table V). Analysis of genotypes B and C showed that the double (K130M/V131I) and triple mutations (N88V/K130M/V131I) were more frequent in genotype C ($P < 0.001$)(Table IV).

Table III. Associations between mutations in the X gene and stage of liver disease

No	Location	Mutation	Mutation frequency (mean ± SD)		*P*-value	Change in prevalence	Association with disease progression
			CH (*n* = 38)	ALD (*n* = 76)			
1.	Trans repression	A12S/T	0.053 ± 0.226	0.000 ± 0.000	0.044*	↓	Novel mutation
		L16P/F	0.053 ± 0.226	0.000 ± 0.000	0.044*	↓	Novel mutation
2.	B cell epitope	S43P	0.000 ± 0.000	0.132 ± 0.340	0.019*	↑	Immune escape mutant
3.	Enhancer II	N88V	0.000 ± 0.000	0.329 ± 0.473	<0.001**	↑	Tumorigenesis
4.	BCP	K130M	0.421 ± 0.500	0.645 ± 0.482	0.026*	↑	Development of HCC
		V131I	0.421 ± 0.500	0.645 ± 0.482	0.026*	↑	Development of HCC

*$P < 0.05$ and **$P < 0.01$ denote significant differences between groups

SD, standard deviation; CH, chronic hepatitis; ALD, advanced liver disease; HCC, hepatocellular carcinoma

Table IV. Associations between double and triple mutations and stage of liver disease

Factor	Mutation frequency (mean ± SD)							
	ALD (*n* = 76)	CH (*n* = 38)	*P* value	HCC	Non-HCC(88)	*P* value	GenB	GenC	*P* value
Double mutation (K130M/V131I)	0.62±0.49	0.42±0.50	0.049*	0.65±0.49	0.52±0.50	0.241	0.40±0.49	0.81±0.39	<0.001**
Triple mutation (N88V/K130M/V131I)	0.29±0.46	0.00±0.00	<0.001**	0.35±0.49	0.15±0.36	0.024*	0.00±0.00	0.52±0.51	<0.001**

*$P < 0.05$ and **$P < 0.01$ denote significant differences between groups

ALD, advanced liver disease; CH, chronic hepatitis; HCC, hepatocellular carcinoma; GenB, genotype B; GenC, genotype C; SD, standard deviation

Table V. Odd ratio analysis

Variable	ALD			*P*-value	HCC			*P*-value
	OR	Lower 95% CI	Upper 95% CI		OR	Lower 95% CI	Upper 95% CI	
K130M	2.495	1.124	5.537	0.025*	1.574	0.633	3.912	0.329
V131I	2.495	1.124	5.537	0.025*	1.574	0.633	3.912	0.329
Double mutation	2.228	1.008	4.925	0.048*	1.725	0.694	4.284	0.240
Triple mutation	2.228	1.008	4.925	0.048*	3.054	1.124	8.300	0.029*

*$P < 0.05$ denotes significant differences between groups

ALD, advanced liver disease; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval

DISCUSSION

This study revealed that most of the HBV strains isolated from Indonesian patients belonged to genotypes B or C, which was confirmed by phylogenetic tree analysis (8, 18, 19). Even though the HBV genotype is commonly specified using the S gene, it appears that a phylogenetic tree constructed for the X gene is also reliable for classifying HBV genotype. Interestingly, some reports have indicated that the X gene is one of the most conserved regions in the HBV genome (20, 21).

It is also unclear how the genotype influences the development of severe liver disease. It has been suggested that genotype C is associated with more severe infection than genotype B (22, 23). The present study showed that the double and triple mutations were dominant in genotype C ($P < 0.001$)(Table IV). Genotype C was associated with a longer phase of hepatitis B e-antigen (HBeAg) positivity and delayed HBeAg seroconversion as compared with genotype B (24). HBeAg seropositivity is an indicator of active viral replication and was shown to be a risk factor for the development of liver cirrhosis and hepatocellular carcinoma. Therefore, delaying HBeAg seroconversion will result in more rapid progression to severe liver disease (25, 26).

The present study was designed to investigate associations between nucleotide changes in the X gene and phenotypic expression in patients with HBV infection to detect mutations associated with advanced liver disease. Of note, mutations in the BCP were significantly more frequent in patients with liver cirrhosis than in patients with chronic hepatitis (Table II). Analysis of advanced liver disease-related mutations also showed that the BCP, especially at T1762 and A1764, is a critical region in the development of advanced liver disease. These modifications would result in the following amino acid changes, K130M and V131I. These amino acid changes are associated with activation of proto-oncogenes and inactivation of the tumor suppressor gene, and may ultimately lead to HBV-related HCC (5, 12, 27). The double mutation at T1762/A1764 was reported to enhance viral replication *in vitro* and that double mutation was significantly higher in advanced liver disease group in our study (Table IV)(21, 28).

This study confirmed that the progression of chronic hepatitis to severe liver disease was associated with the increasing frequencies of the mutations N88V ($P < 0.001$), K130M ($P = 0.026$), and V131I ($P = 0.026$). The mutation S43P is thought to be related to immune escape because it is located in the B cell epitope, which is regions involved in recognition by the host's immune B cells (29, 30). Mutations are more common around immune recognition sites, which suggests that these mutations may be selected as a result of immune pressure and help the virus to escape immune surveillance (31). The Enhancer II mutation N88V was reported to be associated with tumorigenesis and increased risk of advanced liver disease in Taiwanese patients (30, 32). Meanwhile, the hotspot mutation K130M/V131I is well known as a marker of HCC risk, especially in patients with genotypes B, C, or D (33-36). Based on our findings, the decreased mutation frequencies of the novel mutations A12S/T and L16P/F may be related to development of advanced liver disease in Indonesian patients. These mutations were located in the trans repression domain, a key regulatory domain (4, 30, 37). There are two possible explanations. First, these mutations are markers of chronic hepatitis B, because these were dominant in patients with chronic hepatitis as compared with patients with advanced liver disease. Second, this novel mutation may act as a reverse mutation/secondary mutation, such that the modification occurs because of immune pressure or another reason and reversion to the wild-type is associated with worse clinical effects.

We suggest that future studies on the mechanism of HBV X gene point mutations should focus on the biological function of the HBV X protein in association with the development of advanced liver disease. To improve the prediction of advanced liver disease risk and reduce or even avoid the development of advanced liver disease, we think quicker and more accessible methods are needed to detect HBV gene mutations. We also suggest that the sequences submitted to GenBank should be accompanied by additional information, such as the clinical status or phenotype. Such information would allow other researchers to perform reanalysis, recheck, and control for additional information, and prove that the studies are reproducible.

In summary, this study revealed two novel mutations in HBV X gene, A12S/T and L16P/F. Our findings may provide new insights into the mechanism by which multi-site mutations in the X gene may contribute to the development of advanced liver disease. The occurrence of multiple mutations may represent a strategy by which HBV can escape immune surveillance and thus contribute to hepatocarcinogenesis.

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