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ORIGINAL ARTICLE

Association of core promoter mutations of hepatitis B virus and viral load is different in HBeAg(+) and HBeAg(-) patients

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Abstract

AIM: To identify the prevalence of hepatitis B e antigen (HBeAg) and to assess the association of hepatitis B virus (HBV) core promoter mutations and viral load in Indonesian patients.

METHODS: Sixty-four patients with chronic hepatitis, 65 with liver cirrhosis and 50 with hepatocellular carcinoma were included in this study. HBeAg and hepatitis B e antibody (HBeAb) tests were performed using enzyme-linked immunosorbent assay and the mutations were analyzed by sequencing. Viral load was measured by real-time polymerase chain reaction.

RESULTS: Of 179 patients, 108 (60.3%) were HBeAg(-) and 86 (79.6%) of these HBeAg(-) patients had been seroconverted. The A1896 mutation was not found in HBeAg(+) patients, however, this mutation was detected in 70.7% of HBeAg(-) patients. This mutation was frequently found when HBeAg was not expressed (87.7%), compared to that found in HBeAg seroconverted patients (65.1%). The A1899 mutation was also more prevalent in HBeAg(-) than in HBeAg(+) patients (P = 0.004). The T1762/A1764 mutation was frequently found in both HBeAg(+) and HBeAg(-) patients, however, the prevalence of this mutation did not significantly differ among the two groups (P = 0.054). In HBeAg(+) patients, the T1762/A1764 mutation was correlated with lower HBV DNA (P < 0.001). The A1899 mutation did not correlate with HBV DNA (P = 0.609). In HBeAg(-) patients, the T1762/A1764 mutation alone was not correlated with HBV DNA (P = 0.095), however, the presence of either the T1762/A1764 or A1896 mutations was associated with increased HBV DNA (P < 0.001).

CONCLUSION: The percentage of HBeAg(-) patients is high in Indonesia, and most of the HBeAg(-) patients

had been seroconverted. The A1896 mutation was most likely the major cause of HBeAg loss. The T1762/A1764 mutation alone was associated with lower viral loads in HBeAg(+) patients, but not in HBeAg(-) patients.

Key words: Hepatitis B e antibody; Hepatitis B e antigen; Hepatitis B virus; Indonesia; Precore/core promoter mutations; Viral load

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INTRODUCTION

More than 2 billion people are infected with hepatitis B virus (HBV) and 350 million of them are chronic carriers of the virus[1]. Indonesia has a moderate to high endemicity of HBV infection, which is perhaps due to the lack of proper health facilities, poor economical status and less public awareness[2]. HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carriers, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)[3]. Patients with chronic hepatitis B are typically hepatitis B e antigen positive [HBeAg(+)] with detectable HBV DNA in serum. Generally, seroconversion from HBeAg to hepatitis B e antibody (HBeAb) positive correlates with reduced HBV replication in the liver and low infectivity during the natural course of infection[4,5]. In some patients, however, the immune pressure associated with seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage[6].

Most infected patients that are HBeAg(-) harbor HBV variants with mutations in the precore or core promoter region[7]. The predominant precore variation is a G-to-A change at A1896, which creates a premature stop codon and which abolishes the synthesis of HBeAg[8-10]. The most common core promoter mutations involve a two-nucleotide substitution at T1762 and A1764 (T1762/A1764 mutation)[7,11]. Several transfection studies showed that the T1762/A1764 mutation decreased the level of precore mRNA by 50% to 70% and led to reduced HBeAg synthesis[12-14]. A previous study has also demonstrated that HBeAg may be a target antigen on HBV-infected hepatocytes[15]. Failure to produce a target antigen may allow the infected cell to evade immune clearance.

The prevalence of HBeAg(-) patients is likely to vary across geographic areas. The total number of HBeAg(-) chronic hepatitis B patients is higher in the Mediterranean region and is estimated to be up to 33%. However, the prevalence of HBeAg(-) patients who achieved HBeAg seroclearance was higher in Asian patients (36%) than in Mediterranean patients (24%)[16]. Furthermore, HBeAg(-) patients have a higher rate of active liver disease in Asian patients, however, no data from Indonesia is available at present. In a recent study, we analyzed the genotype and core promoter mutations of HBV isolates in Indonesian carriers and patients[17]. The present study identifies the prevalence of HBeAg and assesses the association of HBV core promoter mutations and viral load in Indonesian patients.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 179 HBV-associated liver disease patients, comprising 64 patients with chronic hepatitis B (CH), 65 patients with liver cirrhosis (LC), and 50 patients with HCC. Sera from CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati "Professor Ali Sulaiman", Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Moewardi Hospital, Surakarta, Mataram General Hospital, Mataram, and M. Jamil Hospital, Padang, Indonesia, during the period of May 2006 to March 2010. All sera were persistently seropositive for HBsAg for at least 6 mo. The study was approved by the Institutional Ethics Committee and informed consent was obtained from each patient.

HBeAg and HBeAb tests

HBeAg and HBeAb from all plasma were tested using the MicroLISA™-HBeAg Test and MicroLISA™-HBeAb Test kits (Amgenix, San Jose, CA, USA), according to the manufacturer's instructions.

Analysis of HBV genotype and precore/core promoter mutations

HBV DNA was extracted from 200 μL serum using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80 μL eluted DNA was stored at -70℃ until use. HBV genotype was identified based on S gene sequence or genotype-specific polymerase chain reaction (PCR)[17,18]. Precore and core promoter mutations were analyzed by direct sequencing of the corresponding regions, as described previously[17]. However, for samples in which the precore sequence appeared inconsistent with the HBeAg test, amplification products were inserted into pBluescript Ⅱ SK(+), and at least ten independent clones of each were sequenced.

HBV viral load measurement

HBV DNA was first extracted from 200 μL serum with

Table 1 Demographics and characteristics of patients enrolled in this study

Characteristics	Total	CH	LC	HCC	*P*-value		
					CH *vs* LC	CH *vs* HCC	LC *vs* HCC
n (%)	179 (100.0)	64 (35.8)	65 (36.3)	50 (27.9)	-	-	-
Gender (male/female) (% male)	129/50 (72.1)	38/26 (59.4)	47/18 (72.3)	44/6 (88.0)	0.121	< 0.001	0.040
Age (yr, mean ± SD)	45.8 ± 12.3	39.7 ± 13.3	49.9 ± 10.8	48.4 ± 9.5	< 0.001	< 0.001	0.495
AFP [ng/mL, median (min-max)]	13.2 (0.1-3 295 000.0)	3.2 (0.1-5039.0)	11.8 (1.0-444 718.0)	704.8 (1.2-3 295 000.0)	< 0.001	< 0.001	< 0.001
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	39.5 (7.0-481.0)	66.0 (15.0-297.0)	138.0 (9.0-3618.0)	< 0.001	< 0.001	< 0.001
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47 (6-748)	46 (9-216)	64 (1-860)	0.480	0.481	0.123
AST/ALT	1.28 (0.1-120.6)	0.9 (0.1-2.9)	1.4 (0.2-6.0)	2.1 (0.1-120.6)	< 0.001	< 0.001	< 0.001
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	5.6 ± 2.0	6.1 ± 2.1	5.8 ± 1.4	4.6 ± 2.2	0.334	< 0.001	0.003
All HBeAg(+), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
All HBeAg(-), (%)	108 (60.3)	28 (43.8)	46 (70.8)	34 (68.0)			
HBeAg(+); HBeAb(-), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
HBeAg(-); HBeAb(+), (%)	86 (48.0)	22 (34.4)	40 (61.5)	24 (48.0)	0.002	0.141	0.039
HBeAg(-); HBeAb(-), (%)	22 (12.3)	6 (9.4)	6 (9.2)	10 (20.0)	0.978	0.105	0.098
Genotype							
B, *n* (%)	132 (73.7)	47 (73.4)	48 (73.8)	37 (74.0)	0.958	0.946	0.985
C, *n* (%)	47 (26.3)	17 (26.6)	17 (26.2)	13 (26.0)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of internal control (1 μL/10 μL sample) from ARTUS HBV (Qiagen, Hilden, Germany). From 50 μL of eluted DNA, 20 μL was then quantified by ARTUS real-time PCR assay according to the manufacturer's instructions (Qiagen, Hilden, Germany). The range of HBV DNA detection was 10^1 to 10^5 IU/mL.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). Significance differentiations for continuous variables were analyzed using *t*-test analysis. The categorical variables were analyzed using the Fisher's exact test and χ^2 test. *P*-values of < 0.05 were considered significant.

RESULTS

Characteristics of patients

Patients' characteristics are summarized in Table 1. Of the 179 patients, 129 (72.1%) were male and the male/female ratio was significantly increased from CH to HCC and from LC to HCC, but not from CH to LC. The mean age of all patients was 45.8 ± 12.3 years, and significantly increased from CH to LC and to HCC, but not from LC to HCC. The median level of AST and ALT were 66.0 and 50.0 IU/mL, respectively. The level of AST, but not ALT, was significantly increased according to the severity of liver disease. The mean level of HBV DNA was 5.6 ± 2.0 log_{10} IU/mL, and was significantly lower in HCC (4.6 ± 2.2) than in LC (5.8 ± 1.4) and CH (6.1 ± 2.1). Seventy-one (39.7%) and 108 (60.3%) of patients were HBeAg(+) and HBeAg(-), respectively. The percentage of HBeAg(+) samples was significantly higher in CH (56.3%) than in LC (29.2%) or HCC (32.0). Eighty-six of 179 patients (48.0%) were HBeAg seroconverted, and the percentage of HBeAg seroconversion was higher in LC (61.5%) than HCC (48.0%) and CH (34.4%). There was no difference in HBV genotype prevalence between samples of different clinical diagnosis.

Comparison between HBeAg(+) and HBeAg(-)

Table 2 demonstrates the comparison between HBeAg(+) and HBeAg(-) groups. The mean age of patients was significantly higher in the HBeAg(-) group (49.2 ± 11.7) than in the HBeAg(+) group (40.7 ± 11.5) ($P < 0.001$), indicating a longer period of disease in the HBeAg(-) group. No significant difference in the male/female ratio between the two groups was observed ($P = 0.106$). Surprisingly, there was also no significant difference in ALT levels between the two groups ($P = 0.535$). As shown in Table 3, 79.6% (86/108) of HBeAg(-) patients were HBeAg seroconverted [HBeAb(+)]. There was no significant difference in ALT levels in the samples prior to HBeAg seroconversion [i.e. HBeAg(+), HBeAb(-)] *vs* after HBeAg seroconversion [i.e. HBeAg(-), HBeAb(+)] ($P = 0.200$). However, ALT levels in the HBeAg seroconverted group were significantly higher than those in the group that did not express HBeAg (60.0 IU/L *vs* 43.0 IU/L, $P = 0.023$). The AST/ALT ratio was significantly higher in HBeAg(-) than in HBeAg(+) patients (1.4 *vs* 1.1, $P = 0.022$), suggesting the presence of other factors involved in HBeAg(-) patients. HBV DNA was significantly higher in the HBeAg(+) (6.5 ± 1.8 log_{10} IU/mL) group compared to the HBeAg(-) (5.0 ± 1.9 log_{10} IU/mL) group ($P < 0.001$) (Table 2). The percentage of samples with HBV DNA load ≥ 20 000 IU/mL was much higher in HBeAg(+) patients (91.5%) than in HBeAg(-) patients (68.5%). There was no significant difference in levels of HBV DNA between the HBeAg sero-

Table 2 Comparison of hepatitis B e antigen (+) and hepatitis B e antigen (-) patients

Characteristics	Total	HBeAg(+)	HBeAg(-)	*P*-value
n (%)	179 (100.0)	71 (39.7)	108 (60.3)	-
Gender (male/female) (% male)	129/50 (72.1)	47/24 (66.2)	82/26 (75.9)	0.106
Age (yr, mean ± SD)	45.8 ± 12.3	40.7 ± 11.5	49.2 ± 11.7	< 0.001
AFP [ng/mL, median (min-max)]	13.2 (0.1-3 295 000.0)	10.9 (0.2-230472.0)	19.9 (0.1-3 295 000.0)	0.008
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	55.0 (12.0-635.0)	71.0 (7.0-3618.0)	0.028
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47.0 (1.0-748.0)	52.0 (6.0-860.0)	0.535
AST/ALT	1.3 (0.1-120.6)	1.1 (0.1-30.8)	1.4 (1.4-120.6)	0.022
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	5.6 ± 2.0	6.5 ± 1.8	5.0 ± 1.9	< 0.001
< 20 000 IU/mL, *n* (%)	40 (22.3)	6 (8.5)	34 (31.5)	< 0.001
≥ 20 000 IU/mL, *n* (%)	139 (77.7)	65 (91.5)	74 (68.5)	
Clinical status				
CH, *n* (%)	64 (35.8)	36 (50.7)	28 (25.9)	0.003
LC, *n* (%)	65 (36.3)	19 (26.8)	46 (42.6)	
HCC, *n* (%)	50 (27.9)	16 (22.5)	34 (31.5)	
Genotype				
B, *n* (%)	132 (73.7)	50 (70.4)	82 (75.9)	0.259
C, *n* (%)	47 (26.3)	21 (29.6)	26 (24.1)	

CH: Chronic hepatitis; LC: Liver cirrhosis; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

Table 3 Comparison of patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion

Characteristics	Group 1	Group 2	Group 3	*P*-value		
	HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	1 *vs* 2	1 *vs* 3	2 *vs* 3
n (%)	71 (39.7)	86 (48.0)	22 (12.3)	-	-	-
Gender (male/female) (% male)	47/24 (66.2)	71/15 (82.6)	11/11 (50.0)	0.018	0.171	0.001
Age (yr, mean ± SD)	40.7 ± 11.5	49.8 ± 11.9	46.9 ± 10.6	< 0.001	0.028	0.296
AFP [ng/mL, median (min-max)]	10.9 (0.2-230472.0)	17.1 (0.1-3295000.0)	40.8 (0.4-514412.0)	0.016	0.048	0.731
AST [IU/L, median (min-max)]	55.0 (12.0-635.0)	68.0 (7.0-3618.0)	76.5 (9.0-580.0)	0.017	0.536	0.448
ALT [IU/L, median (min-max)]	47.0 (1.0-748.0)	60.0 (9.0-860.0)	43.0 (6.0-154.0)	0.200	0.173	0.023
AST/ALT	1.1 (0.1-30.8)	1.3 (0.1-120.6)	1.7 (0.5-6.0)	0.091	0.007	0.052
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	6.5 ± 1.8	5.1 ± 1.8	4.5 ± 2.4	< 0.001	< 0.001	0.146
< 20 000 IU/mL, *n* (%)	6 (8.5)	25 (29.1)	9 (40.9)	0.001	< 0.001	0.286
≥ 20 000 IU/mL, *n* (%)	65 (91.5)	61 (70.9)	13 (59.1)			
Clinical status						
CH, *n* (%)	36 (50.7)	22 (25.6)	6 (27.3)	0.001	0.054	0.871
LC, *n* (%)	19 (26.8)	40 (46.5)	6 (27.3)	0.011	0.963	0.104
HCC, *n* (%)	16 (22.5)	24 (27.9)	10 (45.4)	0.442	0.036	0.114
Genotype						
B, *n* (%)	50 (70.4)	66 (76.7)	16 (72.7)	0.369	0.835	0.694
C, *n* (%)	21 (29.6)	20 (23.3)	6 (27.3)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

converted and non-expressing groups ($P = 0.146$) (Table 3), suggesting that HBeAg expression did not affect HBV replication. The percentage of HBeAg(+) samples was high in CH (50.7%) and was less in LC (26.8%) and HCC (22.5%).

Precore/core promoter mutations and HBeAg status

The prevalence of mutations in the precore and core promoter regions was compared between HBeAg(+) and HBeAg(-) patients (Table 4). As expected, the precore A1896 mutation, which is associated with HBeAg expression, was absent in HBeAg(+) patients, but was found in 70.7% of HBeAg(-) patients. Interestingly, the A1896 mutation was frequently found not only in HBeAg non-expressing patients (87.7%), but also in HBeAg seroconverted patients (65.1%). The A1899 precore mutation was found to be more common in HBeAg(-) than in HBeAg(+) patients (34.5% *vs* 10.4%, $P = 0.004$), but there was no significant difference between the HBeAg seroconverted group and the HBeAg non-expressing group (37.2% *vs* 26.7%, $P = 0.116$), suggesting that this mutation contributes to the expression of HBeAg. On the other hand, the T1762/A1764 core promoter mutation was found both in HBeAg(+) (40.8%) and HBeAg(-) (55.6%) patients, and the prevalence of this mutation was not significantly different between the two groups ($P = 0.054$).

Table 4 Precore and core promoter mutations in patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion *n* (%)

	All	Group 1	Group 2	Group 3	Group 2+3	*P*-value			
		HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	HBeAg(-)	1 *vs* 2	1 *vs* 3	2 *vs* 3	1 *vs* (2+3)
T1762/A1764[1]									
Absent	90 (50.3)	42 (59.2)	38 (44.2)	10 (45.5)	48 (44.4)	0.062	0.258	0.915	0.054
Present	89 (49.7)	29 (40.8)	48 (55.8)	12 (54.5)	60 (55.6)				
A1896[2]									
Absent	65 (61.3)	48 (100.0)[3]	15 (34.9)	2 (13.3)	17 (29.3)	< 0.001	< 0.001	0.114	< 0.001
Present	41 (38.7)	0 (0.0)	28 (65.1)	13 (87.7)	41 (70.7)				
A1899[2]									
Absent	81 (76.4)	43 (89.6)	27 (62.8)	11 (73.3)	38 (65.5)	0.003	0.034	0.116	0.004
Present	25 (23.6)	5 (10.4)	16 (37.2)	4 (26.7)	20 (34.5)				
T1762/A1764 and A1896[2]									
Absent	88 (83.0)	48 (100.0)	31 (72.1)	9 (60.0)	40 (68.9)	< 0.001	< 0.001	0.383	< 0.001
Present	18 (17.0)	0 (0.0)	12 (27.9)	6 (40.0)	18 (31.0)				
T1762/A1764 or A1896[2]									
Absent	33 (31.1)	31 (64.6)	2 (4.7)	0 (0.0)	2 (1.9)[4]	< 0.001	< 0.001	0.395	< 0.001
Present	73 (68.9)	17 (35.4)	41 (95.3)	15 (100.0)	56 (98.1)				

[1]Total samples *n* = 179; Hepatitis B e antigen (HBeAg) (+) and hepatitis B e antibody (HBeAb) (-) *n* = 71; HBeAg(-) and HBeAb(+), *n* = 86; HBeAg(-) and HBeAb(-), *n* = 22; [2]Total samples *n* = 106; HBeAg(+) and HBeAb(-) *n* = 48; HBeAg(-) and HBeAb(+), *n* = 43; HBeAg(-) and HBeAb(-), *n* = 15; [3]Three samples had a mixed population of precore stop codon mutation (A1896) and its wild type (G1896); [4]Two samples with a mixed population of basal core promoter mutation (T1762/A1764) and its wild type (A1762/G1764).

However, the presence of either the T1762/A1764 mutation or the A1896 mutation was very high in the HBeAg(-) group (98.1%), [including patients that had seroconverted (HBeAb(+)] (95.3%) and HBeAg non-expressing patients (100.0%), compared to the HBeAg(+) group (35.4% of which had the mutations), indicating these mutations are associated with HBeAg status.

We further analyzed the effect of precore and core promoter mutations on HBeAg expression and viral replication in twenty two HBeAg non-expressed samples. Enough DNA was recovered so that the T1762/A1764 core promoter mutation could be analyzed in all samples, however, the A1896 and A1899 precore mutations could only be analyzed in fifteen samples (Table 5). Either core promoter (T1762/A1764) or precore (A1896 or A1899) mutations were found in all samples, with the exception of three samples from which we were unable to obtain the DNA sequence. Almost all samples demonstrated relatively high HBV DNA, although in some samples the HBV DNA was low, but still detectable. Taken together, these results suggest that precore or core promoter mutations were associated with reduced HBeAg expression, but did not affect HBV replication in groups that did not express HBeAg.

Table 5 Precore and core promoter mutations in samples with hepatitis B e antigen (-) and hepatitis B e antibody (-)

No.	Sample ID	T1762/A1764	A1896	A1899	Serum HBV DNA (log_{10} IU/mL)
1	07.10.068	Yes	No	No	4.59
2	08.70.091	Yes	No	No	4.87
3	08.100.038	Yes	Yes	No	6.43
4	07.10.121	Yes	Yes	No	7.52
5	08.10.002	Yes	Yes	No	7.18
6	09.41.591	Yes	Yes	Yes	6.53
7	09.40.037	Yes	Yes	Yes	5.62
8	10.80.004	Yes	Yes	No	2.78
9	06.10.062	No	Yes	No	3.32
10	08.10.086	No	Yes	No	4.94
11	09.40.033	No	Yes	No	7.28
12	09.80.040	No	Yes	No	4.05
13	09.41.806	No	Yes	Yes	7.42
14	07.10.070	No	Yes	Yes	4.72
15	09.80.037	No	Yes	Yes	6.78
16	08.10.016	Yes	NA	NA	5.21
17	08.10.020	Yes	NA	NA	-0.37
18	08.10.039	Yes	NA	NA	3.10
19	P.X00.34	Yes	NA	NA	-0.54
20	07.10.117	No	NA	NA	1.73
21	07.10.026	No	NA	NA	1.27
22	07.10.173	No	NA	NA	3.48

HBV: Hepatitis B virus.

Precore/core promoter mutations and HBV viral load and ALT

Precore and core promoter mutations correlated with different levels of HBV DNA in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, the T1762/A1764 core promoter mutation correlated with lower HBV DNA levels ($P < 0.001$) (Table 6). The A1899 mutation was not associated with HBV DNA level ($P = 0.609$) and, as expected, no A1896 mutations were detected in the samples. The presence of either the T1762/A1764 or A1896 mutations also correlated with lower HBV DNA levels ($P = 0.011$). On the other hand, in HBeAg(-) patients, T1762/A1764 core promoter, as well as A1896 and A1899 precore mutations were not individually correlated with higher or lower HBV DNA level ($P = 0.095$, 0.231, 0.382, respectively), however, the presence of either the T1762/A1764 muta-

Table 6 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (+) patients

	Serum HBV DNA (log_{10} IU/mL) (mean ± SD) (*n*)		*P*-value	ALT (IU/L) [median (min-max)] (*n*)		*P*-value
	Absent	Present		Absent	Present	
T1762/A1764[1]	7.14 ± 1.46 (42)	5.60 ± 1.99 (29)	< 0.001	43.5 (13.0-748.0) (42)	48.5 (1.0-215.0) (29)	0.806
A1896[2]	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
A1899[2]	7.01 ± 1.25 (43)	6.68 ± 1.53 (5)	0.609	45.5 (1.0-748.0) (43)	55.0 (36.0-92.0) (5)	0.627
T1762/A1764 and A1896[2]	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
T1762/A1764 or A1896[2]	7.33 ± 1.23 (31)	6.33 ± 1.08 (17)	0.011	50.0 (15.0-748.0) (31)	41.5 (1.0-117.0) (17)	0.459

[1]Total samples hepatitis B e antigen (HBeAg) (+) *n* = 71; [2]Total samples HBeAg(+) *n* = 48. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Table 7 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (-) patients

	Serum HBV DNA (log_{10} IU/mL) (mean ± SD) (*n*)		*P*-value	ALT (IU/L) [median (min-max)] (*n*)		*P*-value
	Absent	Present		Absent	Present	
T1762/A1764[1]	4.64 ± 2.00 (48)	5.23 ± 1.79 (60)	0.095	57.0 (6.0-302) (48)	51.5 (6.0-860.0) (60)	0.885
A1896[2]	5.44 ± 1.66 (17)	5.91 ± 1.35 (41)	0.231	46.0 (15.0-860.0) (17)	63.5 (14.0-302.0) (41)	0.321
A1899[2]	5.80 ± 1.29 (38)	5.72 ± 1.76 (20)	0.382	52.0 (14.0-860.0) (38)	58.5 (17.0-302.0) (20)	0.693
T1762/A1764 and A1896[2]	5.59 ± 1.51 (40)	6.17 ± 1.27 (18)	0.138	65.0 (14.0-860.0) (40)	51.0 (17.0-174.0) (18)	0.543
T1762/A1764 or A1896[2]	2.77 ± 4.31 (2)	5.88 ± 1.23 (56)	< 0.001	59.5 (23.0-96.0) (2)	52.0 (42.0-860.0) (56)	0.847

[1]Total samples hepatitis B e antigen (HBeAg) (-) *n* = 108; [2]Total samples HBeAg(-) *n* = 58. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

tion or the A1896 mutation was associated with increased HBV DNA levels ($P < 0.001$) (Table 7). In addition, no correlations between precore and core mutations and serum ALT levels were observed in either the HBeAg(+) or HBeAg(-) patients (Tables 6 and 7).

DISCUSSION

The present study was an epidemiological investigation of the precore and core promoter mutations and their relationship to HBeAg expression levels in Indonesian patients. The majority of patients enrolled in this study were infected with HBV genotype B (73.7%), while the rest were genotype C (26.3%) (Table 1), which is consistent with previous reports[17,19-21]. The prevalence of HBeAg(-) chronic hepatitis B patients was 60.3%, which is similar to other Asian countries[16]. Among the HBeAg(-) patients, 79.6% were HBeAb(+), which meant that they were HBeAg seroconverted (Table 3). Studies in Europe, Asia, and the United States have all reported an increased prevalence of HBeAg(-) chronic hepatitis among HBsAg(+) patients[16]. Our results support previous studies which reported that HBeAg(-) chronic hepatitis B is the most common form of chronic HBV infection in Asia. However, in our study, most of the HBeAg(-) patients had been seroconverted, and the percentage of HBeAg(-) due to abolition of HBeAg synthesis was relatively low (20.4%).

Analysis of the A1896 mutation by direct sequencing demonstrated that all HBeAg(+) samples were wild type (i.e. did not bear the A1986 mutation) (Table 4), however, in our initial sequencing the A1896 mutation was found in three HBeAg(+) samples. The PCR fragments from these samples were cloned into pBluescript Ⅱ SK(+), and sequence analysis of ten clones showed that wild type virus was also present in some isolates. Thus, the presence of these wild type viruses was presumably responsible for HBeAg synthesis. On the other hand, a high percentage of the HBeAg(-) patients bore the A1896 precore mutation. As expected, in HBeAg(-) patients, this mutation was more prevalent in HBeAg non-expressing patients (87.7%) compared to seroconverted patients (65.1%) (Table 4). Nevertheless, the percentage of this mutation in seroconverted patients was relatively high. Since the A1896 mutation creates a premature stop codon which results in abolition of HBeAg synthesis[8-10], seroconverted patients cannot have always had virus with the A1896 mutation. It is believed that precore mutants emerge as a result of selection under immune pressure during the process of HBeAg seroconversion[22-24]. Therefore, at the early stage of infection, the virus might be a wild-type and the A1896 mutation occurs during the process of HBeAg seroconversion. In this study, because the mutation analysis was carried out after HBeAg seroconversion, it is also possible that the A1896 mutation detected in the samples occurred during the process of HBeAg seroconversion. In addition, the prevalence of the A1899 precore mutation was significantly higher in HBeAg(-) than in HBeAg(+) patients ($P = 0.004$), and was also higher in samples before HBeAg seroconversion compared to that after HBeAg seroconversion ($P = 0.003$) (Table 4). These results suggest that the A1899 mutation is associated with expression and seroconversion of HBeAg, which is in accordance with previous studies in Taiwanese patients[25]. Another study from Korea also reported that the A1899 mutation

was frequently found in HBeAg(-) patients, however, the authors found that the A1899 mutation was always accompanied by A1896 mutation[26], which was different to our results.

The frequency of the T1762/A1764 mutation was relatively high both in HBeAg(+) and HBeAg(-) patients, and there was no significant different between the two groups or between patients before and after HBeAg seroconversion. These results suggest that there is no independent association between the T1762/A1764 mutation on HBeAg expression and seroconversion. The presence of both the T1762/A1764 and A1896 mutations correlated with HBeAg expression and seroconversion, but was likely due to the effect of A1896 mutation alone. Although previous studies demonstrated that the core promoter region regulates transcription of the pregenomic and precore RNA and T1762/A1764 mutation suppression, it does not abolish the synthesis of HBeAg, leading to a reduction in HBeAg expression[12,27-29], and we did not observe this phenomenon in our study. Moreover, by measuring HBeAg titer quantitatively, a recent study also reported that the T1762/A1764 mutation reduced the expression of HBeAg[30]. In this study, however, HBeAg analysis was performed qualitatively, which perhaps explains the different results from those of previous studies.

Further analysis of precore and core promoter mutations in HBeAg non-expressed patients revealed that the A1896 mutation alone did not abolish HBeAg synthesis. Theoretically, A1896 mutation creates a premature stop codon which results in abolishment of HBeAg synthesis, however, A1896 was not found in all HBeAg non-expressed patients. Among twenty-two patients with HBeAg(-) and HBeAb(-), we identified the A1896 mutation in fifteen patients of which two did not show the A1896 mutation, but showed the T1762/A1764 mutation (Table 5). To confirm the A1896 mutation in these two samples, we cloned the amplified fragments into the plasmid, and sequenced eighteen clones, and no mutations were found in any of the clones. Furthermore, the A1899 mutation was not found in these samples. These results suggest that the expression of HBAg might not be affected by the A1896 mutation alone, however, further study is needed to investigate other factors involved in HBeAg expression.

In addition to precore and core promoter mutation, HBV variants with point mutations around the Kozak sequence (nucleotides 1809-1812) were also analyzed. Mutations at the Kozak sequence were less common in our samples and were not associated with HBeAg expression (data not shown), which is similar to the results from Korea[31]. Some studies have found that mutations in the Kozak sequence are correlated with HBeAg expression[32-34]. Our study demonstrated that these mutations do not play an important role in the clinical outcome of chronic hepatitis B patients. The differences may be attributable to genetic differences.

Another interesting finding of this study is that the effect of precore and core promoter mutation on HBV viral load was different in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, T1762/A1764 mutation was associated with lower viral load. On the other hand, this mutation was associated with higher viral load in HBeAg(-) patients, including HBeAg seroconverted patients. These results are in accordance with a previous study in Chinese patients[35,36]. It may be postulated that, among individuals who are HBeAg(+), those with both wild-type and mutant viruses have them in different phases of the infection, the former being in the first phase and the latter, at the end of the second phase. These patients experience different immune pressures, resulting in different levels of virus replication. However, those who are HBeAg(-), regardless of core promoter sequence, are in the same phase (the third phase) and experience similar immune pressures, resulting in similar levels of virus replication. If this is the case, it is not difficult to understand why T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but have no effect in HBeAg(-) patients.

In conclusion, the percentage of HBV infected HBeAg (-) patients is relatively high in Indonesia. Most of the HBeAg(-) patients had been seroconverted and the remaining patients did not express HBeAg. A1896 mutation in the precore region was the major cause of the loss of HBeAg expression. T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but are associated with higher viral loads in HBeAg(-) patients.

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COMMENTS

Background

Seroconversion from hepatitis B e antigen (HBeAg)-positive to hepatitis B e antibody (HBeAb)-positive correlates with reduced hepatitis B virus (HBV) replication in the liver and low infectivity during the natural course of infection. However, the immune pressure associated with HBeAg seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage and is known as HBeAg-negative chronic hepatitis B. The aims of the study were to identify the prevalence of HBeAg-negative patients and to assess the association between HBV core promoter mutations and viral load in Indonesian patients.

Research frontiers

To date, there have been no reports on the prevalence of HBeAg-negative chronic hepatitis B in Indonesia. Therefore, it is important to obtain information on the HBeAg status of liver disease patients in Indonesia, and its association with precore and core promoter mutations. In addition, the correlation between precore and core promoter mutations and HBV replication is crucial.

Innovations and breakthroughs

The present study showed that the prevalence of HBeAg-negative chronic hepatitis B in Indonesia is high and most patients had seroconverted. The A1896 mutation was most likely to be the major cause of HBeAg loss. A1899 mutation is also associated with HBeAg-negative and is not always accompa-

nied by A1896 mutation. Furthermore, A1896 was not found in all HBeAg non-expressing patients, two HBeAg(-) and HBeAb(-) patients did not show either the A1896 or A1899 mutation, but had the T1762/A1764 mutation, suggesting that the expression of HBeAg might not be affected by the A1896 mutation alone. Interestingly, the T1762/A1764 mutation was associated with lower viral loads in HBeAg-positive, but not in HBeAg-negative patients.

Applications

The A1896 mutation can be used to predict HBeAg-negative variants in patients who achieve HBeAg seroconversion during disease progression or anti-viral therapy. In addition, the A1899 and T1762/A1764 mutations can be used to predict HBeAg-negative chronic hepatitis B, although there is no A1896 mutation.

Terminology

Core promoter: Part of the HBx gene that regulate the HBe and core gene expression. HBeAg seroconversion: The clearance of HBeAg by the production of HBeAb.

Peer review

The reported work is very intriguing and represents a large undertaking.

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