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Ultradeep Sequencing for Detection of Quasispecies Variants in the Major Hydrophilic Region of Hepatitis B Virus in Indonesian Patients

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Quasispecies of hepatitis B virus (HBV) with variations in the major hydrophilic region (MHR) of the HBV surface antigen (HBsAg) can evolve during infection, allowing HBV to evade neutralizing antibodies. These escape variants may contribute to chronic infections. In this study, we looked for MHR variants in HBV quasispecies using ultradeep sequencing and evaluated the relationship between these variants and clinical manifestations in infected patients. We enrolled 30 Indonesian patients with hepatitis B infection (11 with chronic hepatitis and 19 with advanced liver disease). The most common subgenotype/subtype of HBV was B3/adw (97%). The HBsAg titer was lower in patients with advanced liver disease than that in patients with chronic hepatitis. The MHR variants were grouped based on the percentage of the viral population affected: major, ≥20% of the total population; intermediate, 5% to <20%; and minor, 1% to <5%. The rates of MHR variation that were present in the major and intermediate viral population were significantly greater in patients with advanced liver disease than those in chronic patients. The most frequent MHR variants related to immune evasion in the major and intermediate populations were P120Q/T, T123A, P127T, Q129H/R, M133L/T, and G145R. The major population of MHR variants causing impaired of HBsAg secretion (e.g., G119R, Q129R, T140I, and G145R) was detected only in advanced liver disease patients. This is the first study to use ultradeep sequencing for the detection of MHR variants of HBV quasispecies in Indonesian patients. We found that a greater number of MHR variations was related to disease severity and reduced likelihood of HBsAg titer.

The high mutation rate of hepatitis B virus (HBV), which is attributable to the lack of a proofreading function in its polymerase and the high replication rate of the virus, induces viral diversity, referred to as viral quasispecies. Many studies have demonstrated that the identification of HBV quasispecies may help predict disease progression and therapeutic outcomes (1–3). Accordingly, HBV variants in the quasispecies pool are probably clinically relevant (4).

The surface (S) region used to classify HBV strains is responsible for the expression of hepatitis B surface antigen (HBsAg). HBsAg is the major target for viral neutralization by the immune responses of the host, either naturally or by vaccine-induced antibodies. However, the major hydrophilic region (MHR) located in the central HBsAg, comprising amino acids 100 to 169, contains many discontinuous B-cell epitopes that are recognized by hepatitis B surface antibodies (anti-HBs) (5–7). The exact border of the neutralizing domain of the MHR region is still unclear, although the α-determinant region (amino acids 124 to 147) within the MHR has been known to be strongly involved in antibody binding (8, 9). Moreover, many studies have noted that variations within the MHR can alter the antigenicity of HBsAg and are responsible for (i) the evasion of vaccine-induced antibodies, (ii) the failure of immunoglobulin (HBIg) prophylaxis therapy, and (iii) the diagnostic failure of commercial assays for HBsAg (10). MHR variants that help the virus evade the immune system may influence the persistence of HBV infection, although their contribution to the clinical manifestations of HBV infection remains unknown (11). The MHR variants probably emerged in the circulating viral quasispecies during natural infections.

To date, the most common method used to characterize HBV variants is direct PCR sequencing. However, previous studies have indicated that this method detects variants in the major population only, or ≥20% of the total population (12, 13). Consequently, variants in <20% of the total population are undetected. Recent studies have shown that ultrahigh-throughput next-generation sequencing (NGS) can produce DNA sequences, called reads, in much greater numbers than with direct PCR sequencing (14–16). NGS is also more sensitive and efficient in detecting minor variants, allowing the clarification of their relationships to clinical outcomes (1, 17, 18).

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Hepatitis B infection is moderately to highly endemic in Indonesia, with a carrier rate of 5% to 20% in the general population. Of the Indonesian viruses, some mutations in the HBV genome that are associated with severe liver disease have been reported, including mutations in the basal core promoter region (e.g., A1762T/G1764A and T1753V) (19–21) and the pre-S region (22, 23). Several minor drug-resistant mutations of HBV in Indonesian treatment-naive patients with chronic hepatitis were recently detected using a deep sequencing method (24). However, the prevalence of MHR variants in Indonesian patients has not yet been determined. In this study, we used NGS to detect MHR variants in HBV quasispecies from Indonesian patients with different clinical liver disease status.

MATERIALS AND METHODS

Patients. A total of 30 serum samples from hepatitis B virus-infected patients with different clinical diagnoses were obtained from Hajj and Dr. Mohammad Soewandhie General Hospitals, Surabaya, East Java, Indonesia. Eleven of these samples were from chronic hepatitis B (CHB) patients, and 19 were from CHB patients with advanced liver disease, including 17 patients with liver cirrhosis and two patients with hepatocellular carcinoma. The clinical diagnoses were made at the participating hospitals and were based on clinical, biochemical, and ultrasonographic data supplemented with histopathological data. The study protocol was approved by the ethics committees of Hajj and Dr. Mohammad Soewandhie General Hospitals, and all subjects gave their written informed consent. None of the patients were coinfected with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus.

Serological and biochemical tests and quantification of HBV DNA. The serum HBsAg titer was quantified in all the subjects using the Lumipulse HBsAg-HQ assay, using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse; Fujirebio, Tokyo, Japan). The sensitivity of the Lumipulse HBsAg-HQ assay is 0.005 IU/ml, and the coefficient of variation in this assay is <5.9% for samples with a low concentration of HBsAg (25). Antibodies to the hepatitis B surface antigen (anti-HBs) and hepatitis B e antigen (HBeAg) were assayed with chemiluminescence immunoassays (CLIA) (Architect AUSAB and Architect HBeAg, respectively; Abbott Japan). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using standard procedures. Serum HBV DNA was measured using the TaqMan PCR assay (Cobas TaqMan; Roche Molecular Systems) (lower limit of detection, 2.1 log copies/ml).

DNA extraction and HBV amplification. HBV DNA was extracted from 200 μl of serum using a QIAamp DNA blood minikit (Qiagen, Tokyo, Japan), according to the manufacturer's protocol. The S gene, which includes the MHR, was amplified from each sample with nested-PCR using two primer sets. The first-round PCR primers (outer primer pair) were HB8F (nucleotides [nt] 1824 to 1843 [TTCACCTCTGCCTAATCATC]) and HB6R (nt 1803 to 1784 [AACAGACCAATTTATGCCTA]), and the second-round PCR primers (inner primer pair) were HB2F (nt 414 to 433 [TGCTGCTATGCCTCATCTTC]) and HB3R (nt 1107 to 1084 [AGTTGGCGAGAAAGTGAAAGCCTG]). Nested-PCRs in the first- and second-round PCR were performed as previously described (26). The presence of an amplified product of 693 bp was confirmed by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

Direct sequencing and HBV genotyping. The amplified product from the second round of PCR was purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA), according to the manufacturer's instructions. The PCR products were sequenced using the BigDye Terminator version 3.1 cycle sequencing kit on an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences obtained from direct sequencing and the reference sequences (genotypes A to G) retrieved from GenBank were aligned with the Clustal X software (27). The viral genotype and subtype were identified by phylogenetic analysis of the S gene. A phylogenetic tree was constructed with the neighbor-joining method in the Molecular Evolutionary Genetic Analysis (MEGA) software, version 6.0 (www.megasoftware.net). Bootstrap resampling was performed 1,000 times.

Ultradeep sequencing. An ultradeep sequencer (Genome Analyzer; Illumina, San Diego, CA, USA) was used to detect variations in the MHR of the HBV quasispecies. In the first step, the concentrations of the PCR products of the second round were measured using the Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (catalog no. Q32851; Invitrogen, Carlsbad, CA, USA). In the second step, a library of PCR products (<500 bp) of the viral genome (50 or 0.2 ng, respectively) was prepared using the Nextera DNA sample prep kit (Illumina) or Nextera XT DNA sample prep kit (Illumina), according to the manufacturer's instructions. The PCR products were uniformly sheared to 500-bp fragments using these kits, and the PCR product libraries were mixed with 1% 8 pM PhiX as the control and run on a MiSeq sequencer (Illumina) for paired-end 151-bp sequencing. Finally, imaging-based detection of the fluorescent signals was analyzed using the MiSeq control software (Illumina), and the images were used to develop the sequence data in the FASTQ format.

Sequence read mapping and data analysis. Illumina paired-end sequencing was performed to generate overlapping read pairs (ORP) among relatively short-sequencing-fragment libraries combined with relatively long reads. A quality check and data trimming were necessary before the sequences were assembled using the Genomics Workbench software version 6.0.1 (CLC bio, Aarhus, Denmark). The sequencing results used in this analysis had a read quality of >80% of the consensus sequence estimated from a quality score of 30 (Q30), according to the manufacturer's (Illumina) data. The use of ORP and Q30 filtering was addressed to recover sequencing errors and eliminate all false-positive variants generated by PCR errors during the sequencing process so that these sequence reads could be used to detect viral variants in low abundance with a greater level of confidence. The assembled sequence reads were mapped against the reference HBV genome of genotype B3 and subtype adw (GenBank accession no. AB713527). The viral quasispecies obtained were evaluated in terms of their prevalence in the population. To achieve these objectives, we used the setting "read conflict" in Genomics Workbench. After mapping was complete, any conflicts between the sequence reads were annotated on the consensus sequence and were defined as a nucleotide alteration encoded in at least one of the sequence reads.

The nucleotide alteration resulting in an amino acid change(s) was defined as a variant. The percentage of the variant population was determined from the proportion of nucleotide substitutions that changed the amino acids in coverage depth (total sequence reads per nucleotide position).

The aim of this study was to reflect the nature of the quasispecies variants in the major hydrophilic region *in vivo* as accurately as possible in relation to clinical diagnoses; therefore, we analyzed variants detected in ≥1% of the total viral population. A similar cutoff was applied in previous studies (28, 29).

Statistical analysis. Statistical analyses were performed with SPSS version 22 (IBM Corporation, Armonk, NY, USA). Differences between groups were examined using a *t* test for variables showing a normal distribution or a nonparametric Mann-Whitney *U* test for variables that were not normally distributed. A *P* value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics. Table 1 compares the clinical characteristics of 11 patients with chronic hepatitis and 19 patients with advanced liver disease related to hepatitis B infection. The HBsAg titer was significantly different between the two groups, whereas age, sex, AST levels, ALT levels, HBeAg positivity, and HBV DNA

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TABLE 1 Clinical characteristics of Indonesian hepatitis B virus-infected patients according to clinical diagnosis

Variable[a]	Chronic hepatitis ($n = 11$)	Advanced liver disease ($n = 19$)	P value
Age (mean ± SD) (yr)	52.55 ± 9.417	52.28 ± 11.671	0.949
Sex (no. of males/no. of females)	9/2	15/4	0.852
Genotype (*n*)	B3	B3 (18), B7 (1)	
Subtype	adw1	adw1 (18), ayw1 (1)	
AST level (mean ± SD) (IU/liter)	96.36 ± 138.665	218.40 ± 301.593	0.082
ALT level (mean ± SD) (IU/liter)	69.50 ± 65.870	74.42 ± 66.883	0.795
HBsAg titer (mean ± SD) (IU/ml)	421,520 ± 737,955	21,506 ± 81,294	0.041[b]
HBeAg positive (no. [%])	3 (27)	10 (53)	0.189
Anti-HBs positive (no. [%])	0 (0)	0 (0)	
HBV DNA level (log copies/ml) (mean ± SD)	6.29 ± 1.788	6.81 ± 1.773	0.302
No. receiving telbivudine therapy/total no. of patients (mean duration ± SD [wk])	3/11 (7 ± 7.937)	2/19 (38 ± 14.142)	
Coverage (mean ± SD) (×)	22,745 ± 17,505	39,128 ± 38,135	

[a] AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; anti-HBs, hepatitis B surface antibody.
[b] $P < 0.05$.

level were not (Table 1). Of 30 patients, only 5 patients were treated with telbivudine for <1 year (Table 1).

The S gene was genotyped by direct sequencing, and the genotypes were plotted on a phylogenetic tree (data not shown). A phylogenetic analysis of the published HBV sequences showed that 29 patients were infected with genotype B3 and the adw serotype, and one patient was infected with genotype B7 and the ayr serotype. The ultradeep sequencing method generated HBV sequences for patients with chronic hepatitis and patients with advanced liver disease, providing a mean coverage depth per nucleotide after passing quality control (ORP and Q30) of 22,745× and 39,128×, respectively (Table 1).

MHR variation in different viral populations. Based on the percentage of the variant population in the HBV quasispecies, the MHR variants were selected with a cutoff value of ≥1% of the total viral population and were classified into the following groups: major population, ≥20% of the total population; intermediate population, 5% to <20% of the total population; and minor population, 1% to <5% of the total population. The rate of MHR variation indicates the probability of changes per position. The rates of MHR variation existing in the major and intermediate viral populations were significantly greater in patients with advanced liver disease than in those with chronic hepatitis ($P < 0.05$ and 0.034, respectively). In the minor viral population, the rates of the MHR and α-determinant variations were not significantly different between the patients with chronic hepatitis and those with advanced liver disease ($P = 0.502$ and 0.202, respectively). Only the rate of α-determinant variation in the major viral population was significantly greater in patients with advanced liver disease than that in patients with chronic hepatitis ($P = 0.039$; Fig. 1).

Major viral population of MHR variants. The MHR variants in the major viral population were detected in 8/11 patients with chronic hepatitis (73%) and in 17/19 patients with advanced liver

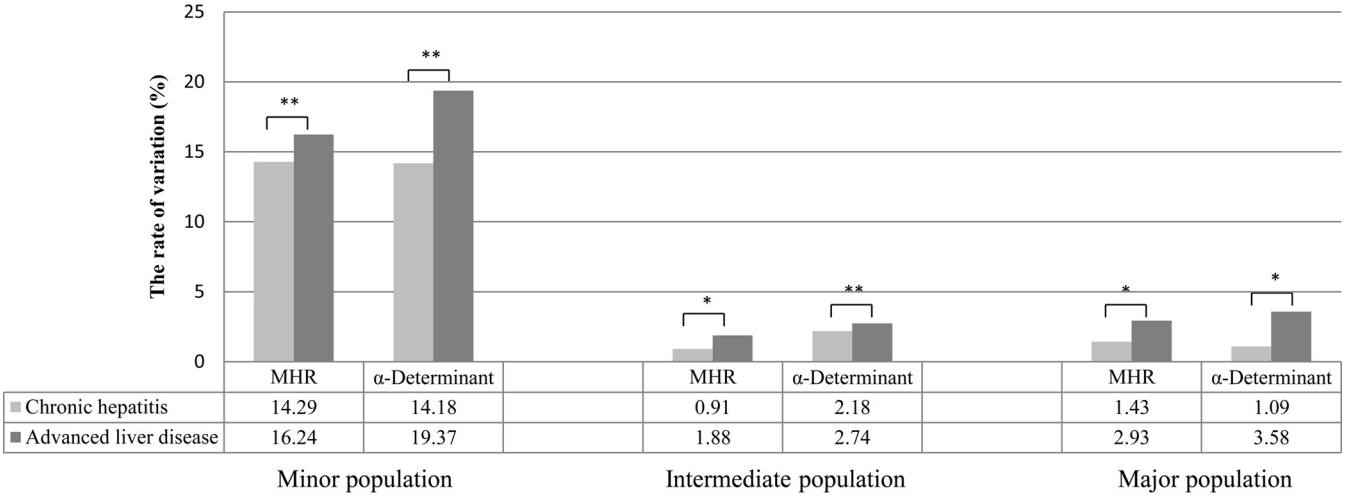

FIG 1 Relationship between the rate of variation in the major hydrophilic region (MHR) and the α-determinant region in different viral populations and severity of liver disease (chronic hepatitis and advanced liver disease). MHR variations in HBV quasispecies were detected by ultradeep sequencing and were divided into the groups according to the percentage of the total variant population: major (≥20% of the total population), intermediate (5% to <20% of the total population), and minor viral population (1% to <5% of the total population). The rate of variation was calculated as (number of changes in the region of each viral population/total number of sites in the region) × 100%. For example, in one patient with 10 amino acid substitutions in the 70-amino-acid MHR existing in the minor viral population, the rate of variation, the calculation was (10/70) × 100% = 14.29%. *, $P < 0.05$; **, $P > 0.05$.

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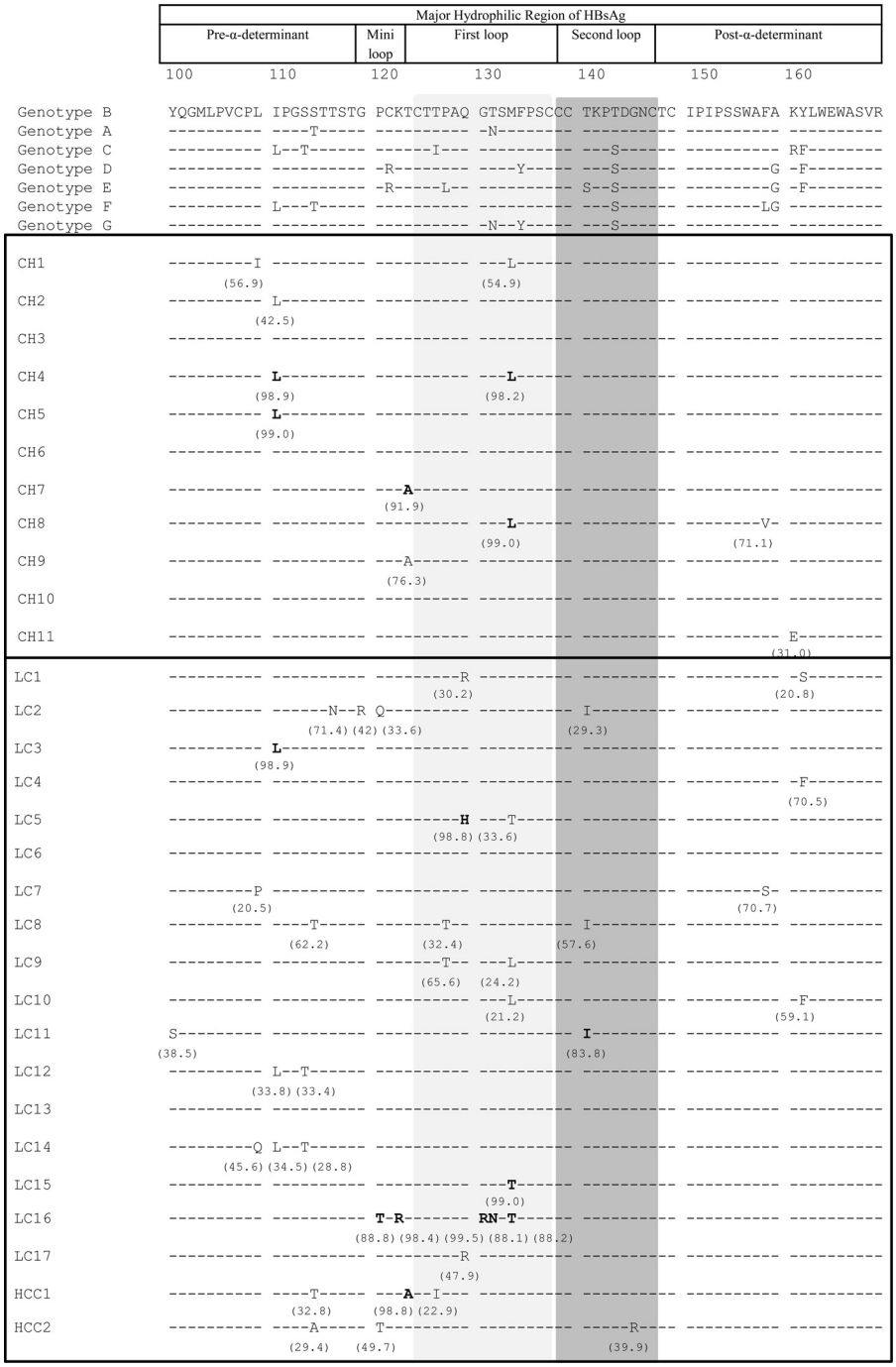

FIG 2 Distribution of the MHR variations in the major viral population. The amino acid sequences of the MHR (amino acids 100 to 169) in samples from patients with chronic hepatitis ($n = 11$) and advanced liver disease ($n = 19$) were compared with the consensus sequences of genotype B and other genotypes (genotypes A, C, D, E, F, and G). The amino acid substitutions were distinguished based on the percentage of the variant population in a quasispecies. Substitutions were categorized as being of the major viral population if they were present in ≥20% of the total population. The major viral population was subdivided into the mixed population (20% to <80% of the total population) or the dominant population (≥80% of the total population [letters in bold type]). CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma. The numbers in parentheses are the % of the variant population.

disease (89%). In this cohort of 25 patients (25/30 [83%]) carrying MHR variants in the major viral population, we detected 50 different amino acid substitutions involving eight positions inside and 12 positions outside the α-determinant, including the first and second loops. Overall, 6/8 positions inside the α-determinant region yielded known immune escape variants (T126I, P127T, Q129H/R, G130R, M133L/T, and G145R). In contrast, only 2/12 positions outside the α-determinant region corresponded to

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TABLE 2 Immune escape variants of the MHR discovered in the major and intermediate viral populations[a]

MHR loop involved in antibody binding (amino acids)	Mutation	Major viral population			Intermediate viral population			Cause (reference[s])
		No. of patients with mutation			No. of patients with mutation			
		Chronic hepatitis ($n = 11$)	Advanced liver disease ($n = 19$)	Mean % of viral population	Chronic hepatitis ($n = 11$)	Advanced liver disease ($n = 19$)	Mean % of viral population	
Mini loop (120–123)	P120T	0	2	69.3	0	3	7.8	HBIg escape (5, 48)[b]
	P120Q	0	1	33.6	0	0		Misdiagnosis (47)
	K122N	0	0		0	1	6.22	Misdiagnosis (46, 49)
	T123A	2	1	89.0	0	0		Misdiagnosis (49)
First loop (124–137)	C124Y	0	0		1	0	13.3	HBIg escape and misdiagnosis (48)
	T125M	0	0		0	1	8.7	Vaccine escape (50)
	T126I	0	1	22.9	0	0		Misdiagnosis (5)
	P127T	0	2	49	0	2	13.3	Vaccine escape (50, 51)
	Q129H	0	1	98.8	0	1	12.1	Vaccine and HBIg escape (5, 51)
	Q129R	0	2	39	0	0		Vaccine escape (49)
	G130R	0	1	99.5	0	0		HBIg escape and misdiagnosis (5, 49)
	M133L	3	2	59.5	0	1	5.8	Vaccine escape, HBIg escape, and misdiagnosis (48, 53)
								Misdiagnosis (49)
	M133T	0	3	73.6	0	0		
Second loop (138–147)	C138Y	0	0		1	0	5.4	Misdiagnosis (49)
	C139Y	0	0		1	0	7.5	HBIg escape and misdiagnosis (48, 56)
	D144A	0	0		0	1	6.7	Vaccine escape, HBIg escape, and misdiagnosis (48, 52)
	G145R	0	1	39.9	1	5	10.4	Vaccine escape, HBIg escape and misdiagnosis (5, 51)
	C147F	0	0		0	1	7.1	Misdiagnosis (49)

[a] MHR, major hydrophilic region.
[b] HBIg, hepatitis B immunoglobulin.

known escape variants (P120T/Q and T123A) (Fig. 2). The immune escape variants of the MHR region in the major viral population were more frequently observed in the first loop of the α-determinant (Table 2).

Although all samples belonged to genotype B, several MHR variants related to the genetic background of other genotypes were also found, including L110 (wild-type strain of genotypes C and F), T113 and I126 (wild-type strains of genotype C), T114 (wild-type strain of genotypes A and F), N131 (wild-type strain of genotypes A and G), and F161 (wild-type strain of genotypes C, D, and E). Notably, several MHR variants that are known to impair HBsAg secretion were also detected, including G119R, Q129R, T140I, and G145R. These variants in the major viral population were detected in patients with advanced liver disease only (Fig. 2).

The quantification of the variant population detected using ultradeep sequencing describes the true proportion of quasispecies variants. Therefore, MHR variants in the major viral population can be considered in terms of their origins, i.e., preexisting variants or those that are likely to be selected by the immune response. Variants existing in the dominant population (i.e., ≥80% of the total viral population) are known as preexisting variants. In contrast, variants in the mixed populations (20% to <80% of the total population) may emerge by immune selection. In this study, 15/50 amino acid substitutions in the major viral population at positions 110, 120, 122, 123, 129, 130, 131, and 133 were classified as the dominant population in some patients (Fig. 2). The presence of MHR variants in the mixed population (≥20% to 80% of the total population) correlated positively with disease severity (Fig. 3).

Intermediate viral population of MHR variants. The intermediate viral population of MHR variants was found in 4/11 (36%) patients with chronic hepatitis and in 15/19 (79%) patients with advanced liver disease (Fig. 4). A total of 32 MHR variants in the intermediate population were detected in 19/30 (63%) patients. Overall, 19/32 variants were located inside the α-determinant region at 11 positions, but only eight positions contained mutations associated with immune escape (C124Y, P127T, Q129H, M133L, C139Y, G144A, G145R, and C147F; Fig. 4). P120T and K122N, which are associated with immune escape and are located outside the α-determinant, were also found in the intermediate viral population (Fig. 4). The most frequent variant in the intermediate viral population was G145R, which was detected in a slightly greater proportion of patients with advanced liver disease (5/19 [26%]) than with chronic hepatitis (1/11 [9%]). A high frequency of immune escape variants in the intermediate viral population occurred in the second loop of the α-determinant (Table 2).

Minor viral population of MHR variants. In this study, minor variants in the MHR corresponding to immune escape variants and stop codons were also detected with ultradeep sequencing. As shown in Table 3, most of these immune escape

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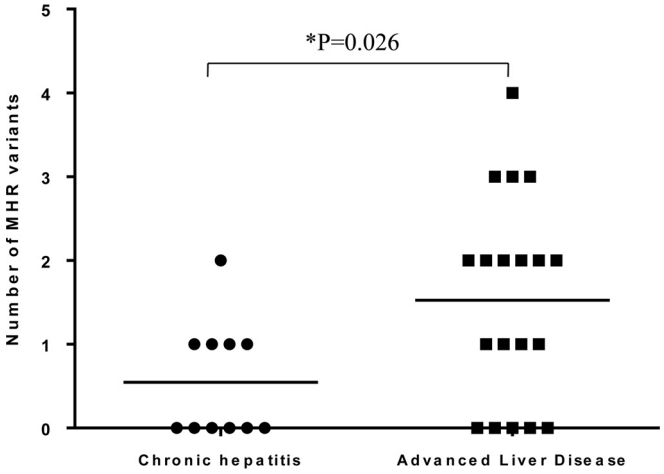

FIG 3 Frequency of MHR variants in the mixed population (present in 20% to <80% of the total viral population). The number of MHR variants that exist in a mixed population was compared between samples from patients with chronic hepatitis ($n = 11$) and advanced liver disease group ($n = 19$). MHR, major hydrophilic region. *, $P < 0.05$.

variants were located in the α-determinant region. The most frequent stop codons were W156*, W163*, and W165*, which were detected in 1% to 3.18% of the total viral population (Table 3).

DISCUSSION

The present study demonstrates that almost all the HBV strains (97%) isolated in Surabaya, East Java belonged to subgenotype B3 and subtype adw. These results are consistent with previous studies showing that HBV genotype B3 and subtype adw are predominant in western Indonesia (30–33). Some studies have shown that HBV subtype adw correlated strongly with vaccine escape mutants in Singapore (34) and surface gene variations in young Taiwanese patients with hepatocellular carcinoma (HCC) (35). In the current study, we focused on the MHR of the S gene because of the highly antigenic region of HBsAg. Variations in the MHR can alter the antigenicity of HBsAg, permitting the virus to evade neutralizing anti-HBs (8, 36, 37).

The severity of liver disease in the patients in this study was significantly associated with a reduced HBsAg titer, consistent with the results of a previous study (38). HBeAg positivity also tended to be greater in patients with advanced liver disease than in patients with chronic hepatitis (10/19 [53%] versus 3/11 [27%], respectively). Overall, the mean HBV DNA level was not different between CHB and advanced liver disease patients. Meanwhile, the HBV DNA level in the HBeAg-positive group was found to be significantly higher than that in the HBeAg-negative group (data not shown). The presence of HBeAg indicates more active viral replication and therefore greater infectivity and risk of disease progression (39). The present study suggests that the presence of HBeAg may related to severe liver disease in the Indonesian population. The clinical manifestations were probably influenced by the duration of HBV infection (35), because there was no difference in the mean ages of the patients with chronic hepatitis or advanced liver disease in this study. The duration of infection is defined as the exposure term as a consequence of vertical or horizontal transmission.

Understanding the profile of HBV quasispecies within the MHR of HBsAg in an population endemic for the virus, like Indonesia, is important and may help researchers make decisions regarding the design of vaccines or immunodiagnostic reagents and develop therapeutic strategies using HBIg in the future. This study was the first attempt to detect the MHR variants in HBV quasispecies from Indonesian patients infected with hepatitis B virus at different clinical stages of the disease. We used ultradeep sequencing to achieve this objective. Recent studies have demonstrated that ultradeep sequencing is a more sensitive and effective tool for detecting variants in viral quasispecies than conventional direct PCR sequencing, because ultradeep sequencing can identify low-abundance variants (1, 17, 18, 40). Moreover, ultradeep sequencing is not only useful for the detection but also the quantification of variants in the viral quasispecies pool (41, 42). Therefore, the true prevalence of variants in viral quasispecies that may be clinically relevant can be determined with ultradeep sequencing.

In order to improve the read accuracy of ultradeep sequencing to study the minor-variant population, we applied overlapping paired-end reads and Q30 for read filtering, as proposed in a previous study (43). Using ultradeep sequencing, we were able to include MHR variants corresponding to proportions of 1% to 100% of the total viral population. Notably, we found that the rates of MHR variation that was present in the major (≥20% of the total population) and intermediate (5% to <20% of the total population) viral populations of quasispecies were significantly greater in patients with advanced liver disease than those in patients with chronic hepatitis. These results suggest that the accumulation of MHR variants increases with the severity of liver disease. Similarly, it was previously reported that the frequency of variations in the MHR was greater in patients with advanced liver disease than that in patients with less severe disease (44, 45). Currently, the extent of the accumulation of MHR variants during disease progression is unclear. However, there are two possible explanations. First, patients may be infected with HBV carrying accumulated variants, contributing to persistent infection and the development of HCC. Second, additional variants are generated during viral replication or persist because of immune escape, contributing to the persistent infection and disease progression. The precise role of MHR variations in disease progression requires further investigation.

In the present study, the presence of MHR variants in the major and intermediate viral populations was presumably associated with increasing disease severity. Several variants were previously documented as being immune escape variants (46–52) and were predominantly located in the α-determinant region (amino acids 124 to 147). Some of the most frequent immune escape-related variants detected in this study were P120Q/T, T123A, P127T, Q129H/R, M133L/T, and G145R. Particularly, T123A and M133L were previously identified in Indonesian blood donors with occult HBV (53). Both variants were associated with diagnostic failure (48, 49). We detected T123A and M133L in patients with chronic hepatitis and in patients with advanced liver disease. In contrast, P120Q/T, P127T, Q129H/R, M133T, and G145R were found only in patients with advanced liver disease. Importantly, M133T and G145R are not only related to immune escape (5) but might also be implicated in the development of HCC (49, 54, 55). Amino acid changes at positions 120 (P120T/Q) and 129 (Q129H/R) have been detected as vaccine/HBIg escape mutants in newborn babies with HBsAg-positive mothers (56, 57) and cause false-negative

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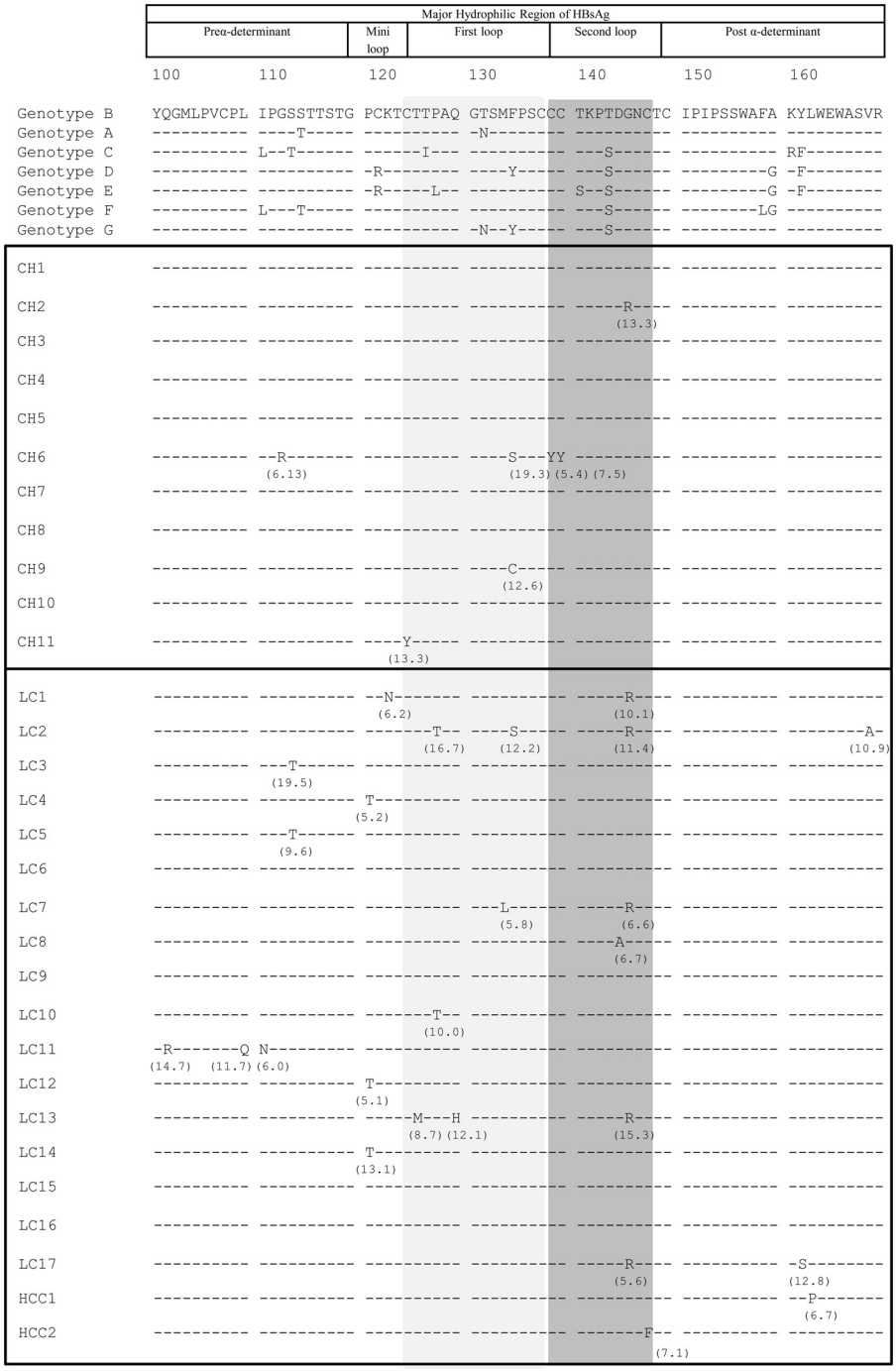

FIG 4 Distribution of the MHR variations in the intermediate viral population. The amino acid sequences of the MHR (amino acids 100 to 169) in samples from patients with chronic hepatitis ($n = 11$) and advanced liver disease ($n = 19$) were compared with the consensus sequences of genotype B and other genotypes (genotypes A, C, D, E, F, and G). The amino acid substitutions were distinguished based on the percentage of the variant population in a quasispecies. Substitutions were categorized as being of the intermediate viral population if they were present in 5% to <20% of the total population. CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma. The numbers in parentheses are the % of the variant population.

results in some commercial assays for HBsAg (58). The naturally occurring P120T variant has also been detected in some unvaccinated and treated patients (59, 60). The variant P127T was reported to reduce the binding of HBsAg to monoclonal antibodies (61). Several variants in the MHR found in this study were the genetic background of HBV genotypes. The variant L110 major-variant population, which is wild-type strain of genotype C, was detected prominently in genotype B of our study population and did not correlate with clinical status. Nevertheless, a variant of I110, which is the wild-type strain of genotype B, was predominant in HBeAg-negative patients with genotype C (62).

Some MHR variants (e.g., G119R, C124Y, I126S, Q129R,

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TABLE 3 Immune escape variants and stop codons of MHR in the minor viral population[a]

No.	Sample[b]	Immune escape variants and stop codons in the MHR
1	CH1	T123A (1.07), C149R (1.12), **W165[c] (1.84)**
2	CH2	T123A (1.17), T131I (3.16), F134L (1.05)
3	CH3	T118A (1.06), C124R (1.05), Q129H (3.42), M133L (2.97), F134L (1.43), N146S (1.04)
4	CH4	F134L (1.03)
5	CH5	T118A (1.03)
6	CH6	T123A (1.03), C124Y (2.38), M133I (1.51), C137Y (2.5), G145R (1.97), C147Y (1.44), T148A (1.12), **W156* (1.69)**, **W163* (1.12)**
7	CH7	F134L (1.03), **W163* (3.18)**
8	CH8	
9	CH9	G130R (1.24), C138Y (1.52), C139Y (1.55), G145 (1.07), **W156* (1.06)**, Y161C (1.55)
10	CH10	F134L (1.08)
11	CH11	T126I (1.11)
12	LC1	
13	LC2	T123A (1.06), M133L (2.57)
14	LC3	Q129H (1.03), F134L (1.63), C137R (1.16), N146S (1.19), F158Y (1.27), **W165* (2.12)**
15	LC4	C124R (1.95), M133L (1.41), F134L (1.15), G145R (1), **W165* (1)**
16	LC5	T118A (1.23), D144A (1.03), G145R (1.59), T148H (1.47)
17	LC6	C124Y (1.01), T125M (1.5), M133I (3.54), F134L (1.22), C138Y (2.27), K141E (1.1), C147Y (1.21), **W163* (2.95)**
18	LC7	P120Q (1.74), T126S (3.94), T131N (2.61), C138Y (1.63), C139Y (2.55)
19	LC8	F134L (1.1), C138Y (1.92), C139Y (2.29), **W156* (1.06)**
20	LC9	F134L (1.16), C139Y (1.41)
21	LC10	T116N (3.56), C124Y (1.78), T126S (4.96), F134L (1.34), P135S (2.36), C138Y (3.74), C139Y (2.19), G145E (2.28), F158S (2.46)
22	LC11	T123A (3.54), T126A (2.18), C138Y (1.06), C147Y (1.28)
23	LC12	T118S (1.01), T123A (1.68)
24	LC13	G130R (1.19), T131N (1.57), M133L (2.31), F134L (1.16), C138Y (1.15), C139Y (2.04)
25	LC14	T123A (1.34), T131A (1.04), F134L (1.31), C139Y (1.39)
26	LC15	F134L (1.37)
27	LC16	C138Y (1.17), C139Y (1.15), **W156* (1.02)**
28	LC17	T115A (1.22), T123A (1.22), P127S (2.07), M133T (1.21), F134L (1.36), C139Y (1.6)
29	HCC1	
30	HCC2	T123A (3.53), Q129H (1.87), M133L (2.97), **W156* (3.18)**

[a] Immune escape variants or stop codons in the MHR region were categorized in the minor viral population if present in 1% to <5% of the total population.
[b] CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.
[c] Stop codons are indicated in boldface. The numbers inside parentheses are % of the number of viral population in the quasispecies pool.

S136P, C139R, T140I, K141E, D144A, and G145R) can impair the secretion of HBsAg particles and virion secretion. Furthermore, the accumulation of HBsAg or virions in hepatocytes can induce endoplasmic reticulum stress, promote inflammation, and eventually lead to more severe liver disease (63). Consequently, these variants seem to be associated with a reduced likelihood of HBsAg production. Accordingly, the titer of HBsAg in our patients with advanced liver disease was significantly lower than that in patients with chronic hepatitis. Furthermore, some MHR variants that impair HBsAg secretion in the major viral population were often detected in patients with advanced liver disease but not in patients with chronic hepatitis. Therefore, future studies should examine whether an increased number of MHR variants is associated with reduced serum HBsAg titers.

The major viral population of MHR variants obtained from ultradeep sequencing can be differentiated into a mixed population (20% to <80% of the total population) and a dominant population (≥80% of the total population). Variants in the dominant population, defined as variants replacing the wild-type strain in the major viral population, may be acquired at the onset of HBV infection. This concept is supported by the results of previous studies, which have shown that the replacement of the wild-type strain with a variant may take >50 years (64–66). Some immune escape variants were identified in the dominant population, which indicates that these variants are relatively stable in the absence of the wild-type strain. Previous reports have shown that the G145R variant is stable over time (67, 68). However, infection with the dominant population of immune escape variants increases the risk of horizontal transmission in the general Indonesian population, even in vaccinated individuals. In contrast, a mixed population, defined as a mixture of wild-type and variant strains in the major viral population, may be selected by the immune responses of the host. It seems to us that the high frequency of MHR variants in a mixed population might be driven by interactions between the virus and the immune responses of the host during disease progression.

Interestingly, this is the first study to detect MHR variants in the intermediate viral population and to demonstrate their correlation with the severity of liver disease. The prevalence of G145R in the intermediate viral population, the most important and best documented immune escape variant, was slightly greater in patients with advanced liver disease than that in patients with chronic hepatitis. Although the abundance of this variant was low in the intermediate viral population, the emergence of G145R mutations should be considered and monitored in patients with advanced liver disease undergoing HBIg therapy because of its impact on the efficacy of immunotherapy.

The major viral population of immune escape variants was

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predominantly observed in the first loop of the α-determinant (amino acids 124 to 137), consistent with previous studies (5, 9, 45). However, the intermediate viral population of immune escape variants was predominantly observed in the second loop (amino acids 138 to 147). To note, the immune escape variants in the second loop are mostly induced by active and/or passive immunity (9, 69). Although the prevalence of MHR variants in the minor viral population was not associated with disease severity, the immune escape variants and stop codons in the MHR that were detected in the minority population with ultradeep sequencing were also identified in this study. The most common stop codons were W156Stop, W163Stop, and W165Stop, which were detected in 1% to 3.18% of the viral population. Notably, W156Stop and W163Stop are associated with immune therapy failure and HBV infectivity (70, 71). To our knowledge, W165Stop has not been reported elsewhere. The clinical relevance of these minor variants must be confirmed in follow-up studies.

In summary, the present study demonstrates an association between the accumulation of MHR variants present in ≥5% of the viral population (intermediate and major viral populations) and disease severity. The increased HBV quasispecies in the MHR may be involved in the progression of liver disease as a consequence of immune evasion or impaired HBsAg secretion. The true proportion of immune escape variants in HBV quasispecies can be determined with ultradeep sequencing, and the results may be useful in predicting the efficacy of vaccine-induced immunity or immunotherapy in the future.

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