Jpn. J. Infect. Dis., 70, 647–655, 2017

Original Article

Profile of Mutations in the Reverse Transcriptase and Overlapping Surface Genes of Hepatitis B Virus (HBV) in Treatment-Naïve Indonesian HBV Carriers

Laura Navika Yamani[1], Yoshihiko Yano[4,5]*, Takako Utsumi[1,4], Widya Wasityastuti[2], Hanggoro Tri Rinonce[3], Dewiyani Indah Widasari[3], Juniastuti[1], Maria Inge Lusida[1], Soetjipto[1], and Yoshitake Hayashi[4]

[1]Institute of Tropical Disease, Airlangga University, Surabaya; [2]Department of Physiology; [3]Department of Anatomical Pathology, Faculty of Medicine, Dr. Sardjito Hospital, Gadjah Mada University, Yogyakarta, Indonesia; [4]Center for Infectious Diseases; and [5]Department of Gastroenterology, Kobe University Graduate School of Medicine, Hyogo, Japan

SUMMARY: Mutations in the reverse transcriptase (RT) region of the hepatitis B virus (HBV) genome are an important factor in low therapeutic effectiveness. Nonetheless, the prevalence of these mutations in HBV strains isolated previously in Indonesia has not been systematically examined. Therefore, in this study, we investigated the profile of mutations in the RT region and the associations of these mutations with amino acid changes in the surface protein in the virus of treatment-naïve Indonesian HBV carriers. Overall, 96 sequences of the full-length Indonesian HBV genomes (genotype B, $n = 54$; genotype C, $n = 42$) were retrieved from the National Center for Biotechnology Information. Naturally occurring primary and/or compensatory drug resistance mutations were found in 6/54 (11.1%) genotype B strains and in 1/42 (2.4%) genotype C strains. The potential mutations underlying resistance to a nucleos(t)ide analog and/or pretreatment mutations were more frequent in both genotypes but more frequent in genotype C strains than in genotype B strains. The A–B interdomain region in the RT gene was more frequently mutated in genotype C than in genotype B (3.51 ± 2.53 vs. 1.08 ± 1.52, $P < 0.001$). Knowledge about the mutational profiles of the RT gene and changes in the surface protein may help clinicians to select the most appropriate antiviral drug and vaccination or HBV immunoglobulin regimen for management of HBV infection in Indonesia.

INTRODUCTION

Nucleos(t)ide analogs (NAs), such as lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir, inhibit replication of hepatitis B virus (HBV) and prevent viral hepatitis. Nonetheless, antiviral therapy with these drugs is often ineffective because of the emergence of antiviral-drug resistance mutations, located in the HBV reverse transcriptase (RT) gene during long-term treatment (1). Therefore, antiviral-drug resistance mutations have even been reported in HBV isolated from treatment-naïve patients (2,3)

HBV RT mutations are classified into 4 categories: primary drug resistance mutations, secondary, or compensatory mutations, putative NA resistance (NAr) mutations, and pretreatment mutations (4). Primary drug resistance mutations are directly responsible for NA resistance. These mutations affect positions rt169, rt181, rt184, rt194, rt202, rt204, rt236, and rt250 (5,6). Secondary or compensatory mutations are amino acid (aa) substitutions that restore functional defects in RT activity associated with primary drug resistance because they can compensate for the fitness loss. Mutations at positions rt80, rt173, and rt180 belong to this category (7). Some putative NAr mutations have been reported (8-11). Unfortunately, not all the effects of these mutations have been verified experimentally in vitro. Other aa substitutions that exist before initiation of tretment are called pretreatment mutations (12). The relations between these mutations and the emergence of antiviral-drug resistance have not been clarified.

The HBV RT mutations are located at 3 sites: the functional RT domain, the A–B interdomain, and the non A–B interdomain. The RT gene overlaps completely with the envelope (surface, S) gene (8). Hence, RT mutations may result in changes in the aa sequence encoded by the S gene. For example, some RT mutations in the A–B interdomain may cause aa substitutions in and around the "a" determinant. Such mutations may reduce the binding affinity of neutralizing antibodies, causing vaccine escape and/or hepatitis B immunoglobulin (HBIg)-selected escape (6,10). The frequency of the RT mutation was reported to vary among HBV genotypes and treatment-naïve patients in many countries although the clinical significance of some mutations is unclear (13).

Indonesia is a country with moderate to high endemicity of HBV infection, with a carrier rate of 5%–20% in the general population (14). HBV genotype B is predominant in western regions of Indonesia, while genotype C is dominant in eastern regions (15,16). It has been shown that 2 mutations in the basal core promoter region, A1762T/G1764A and T1753V, are present in

Received March 13, 2017. Accepted August 3, 2017.
J-STAGE Advance Publication October 31, 2017.
DOI: 10.7883/yoken.JJID.2017.078
*Corresponding author: Mailing address: Center for Infectious Diseases, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan. Tel: +81-78-382-6305 (5700), Fax: +81-78-382-6309: E-mail: yanoyo@med.kobe-u.ac.jp

59.5% and 40.5% of patients, respectively, and are associated with severe liver disease in Indonesian population (17). The prevalence of pre-S mutations has also been reported (18,19). Nevertheless, the RT region has not been fully characterized.

The aim of this study was to determine the naturally occurring mutations in the RT and S genes of HBV strains isolated from treatment-naïve Indonesian HBV carriers. The prevalence rates and profiles of the mutations were also analyzed and compared between the genotypes B and C strains.

MATERIALS AND METHODS

Data collection on HBV genomes in GenBank: To identify naturally occurring mutations in the RT and S genes of HBV strains from Indonesia, 96 HBV whole genome sequences were retrieved by searching GenBank (Table 1; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov> with the keywords "hepatitis B virus", "complete genome", and "Indonesia". All the sequence data in this study are available under an open source license (or NCBI database).

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. As described in the original reports, all the sequences were obtained from treatment-naïve HBV carriers, including asymptomatic carriers (blood donors and hemodialysis patients) and patients with an HBV-related liver disease (Table 1).

Table 1. Whole genome sequences of HBV strains isolated from Indonesian carriers retrieved from the GenBank database

Accession no. (n = 96)	Genotype	Origin	Population	Ref.
AB033554, AB033555	B	Jakarta, Padang	Blood donors	(20)
M54923	B	Manado	N/A	(21)
EF473971, EF473972, EF473973, EF473974, EF473975, EF473976, EF473977	B	East Nusa Tenggara	The Javanese population of West Indonesia, Alorese, East Sumbanese, and Kodi of West Sumba populations, Indonesian Chinese living in Java	(22)
AP011084, AP011085, AP011086, AP011087, AP011089, AP011088, AP011090, AP011091, AP011092, AP011093, AP011094, AP011095, AP011096	B	Pontianak, Jakarta, Banjarmasin, Kendari, Makassar, Waikabubak, Manado, Tahuna, Ternate, Biak, Jayapura, Mataram, Bima, Maumere, Denpasar, Dili, Kupang	Subjects with HBsAg living in 28 cities on 15 Indonesian islands	(15)
AP011097, AP011098, AP011099, AP011100, AP011101, AP011102, AP011103, AP011104, AP011105, AP011106, AP011107, AP011108	C			
AB493827, AB493833, AB493834, AB493835, AB493836	B	Papua	Blood donors in Papua, including Papuan and non-Papuan inhibitants	(23)
AB493837, AB493838, AB493839, AB493840, AB493842, AB493847	C			
AB540582	B	East Nusa Tenggara	Blood donors in Kupang, Timor Island of Nusa Tenggara	(24)
AB540583, AB540584, AB540585	C			
AB560661, AB560662	C	Papua	Blood donors in highland and low land Papua	(25)
AB554017	B	Papua	Indigenous inhabitants in Papua	(26)
AB554014, AB554015, AB554018, AB554019, AB554020, AB554021, AB554022, AB554025	C			
JQ429082	B	N/A	N/A	(18)
GQ358136, GQ358137, GQ358138, GQ358139, GQ358140, GQ358141, GQ358142, GQ358143, GQ358144, GQ358145, GQ358146, GQ358147, GQ358148, GQ358149, GQ358150, GQ358151, GQ358152	B	Java, North and West Sumatra, South Sulawesi, Papua, Ternate, West and East Flores, Alor, Mentawai, Nias, Lombok, West and East Sumba, Moluccas, Papua	Asymptomatic carriers, HBV-related liver disease patients, and blood donors	(16)
GQ358153, GQ358154, GQ358155, GQ358156, GQ358157, GQ358158	C			
AB644281, AB644283, AB644284, AB644286, AB644287	C	Papua	Indigenous inhabitants in Nabire, including senior high school students and outpatients without liver disease who visited Bumi wonorejo Public Health Center or Santo Rafael Clinic in Nabire, suffering from malaria, common cold, gastroenteritis, or tuberculosis	(27)
AB713527, AB713528, AB713529, AB713530, AB713531, AB713532	B	Yogyakarta	Hemodialysis patients	(28)

N/A, not available (information regarding the study population was not included in the reference or in the GenBank database).

The study protocol was approved by the Ethics Commitees of Kobe University, Japan, and of the Institute of Tropical Disease, Airlangga University, Indonesia.

Phylogenetic tree analysis: The genotypes were determined based on the information contained within the references and confirmed by phylogenetic analysis (Fig. 1). Multiple alignment was carried out using the Clustal X software <http://www.clustal.org/>. A phylogenetic tree was constructed by the neighbor-joining 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 in the Molecular Evolutionary Genetics Analysis (MEGA) software, ver. 4.0.2 <http://megasoftware.net/>.

Analysis of aa substitutions: The aa substitutions caused by mutations in the RT and S region were analyzed by aligning and comparing the sequences with one of sequences deposited in GenBank using web-based searches examining sequence similarities <http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml>. All statistical analyses were performed in the SPSS software ver. 22.0 (IBM, Armonk, NY, USA). Statistical significance was determined by the parametric *t*-test for normally distributed data, or by the non-parametric Mann-Whitney *U* test for non-normally distributed data. Data with P values < 0.05 were considered statistically significant.

RESULTS

Characteristics of the retrieved genome sequences: The 96 whole-genome sequences of HBV strains represented some regions of Indonesia (Table 1). The retrieved HBV strains from treatment-naïve Indonesian carriers were analyzed to identify RT and S mutations. Of the 96 strains, 54 (56.3%) were classified as genotype B and 42 (43.8%) as genotype C (Fig. 1 and 2). The subgenotype distributions of both genotypes were confirmed by phylogenetic analysis and are shown in Fig. 2.

Analyses of RT mutations: Sequences (1,032 bp) that included the complete RT gene and all known mutations associated with NA resistance were analyzed in both genotypes. The RT mutations were classified into 4 categories, as previously described (4). The pattern, frequencies, and significance of differences in the RT mutations between the 2 genotypes are shown in Table 2.

Primary mutations underlying NA resistance (category

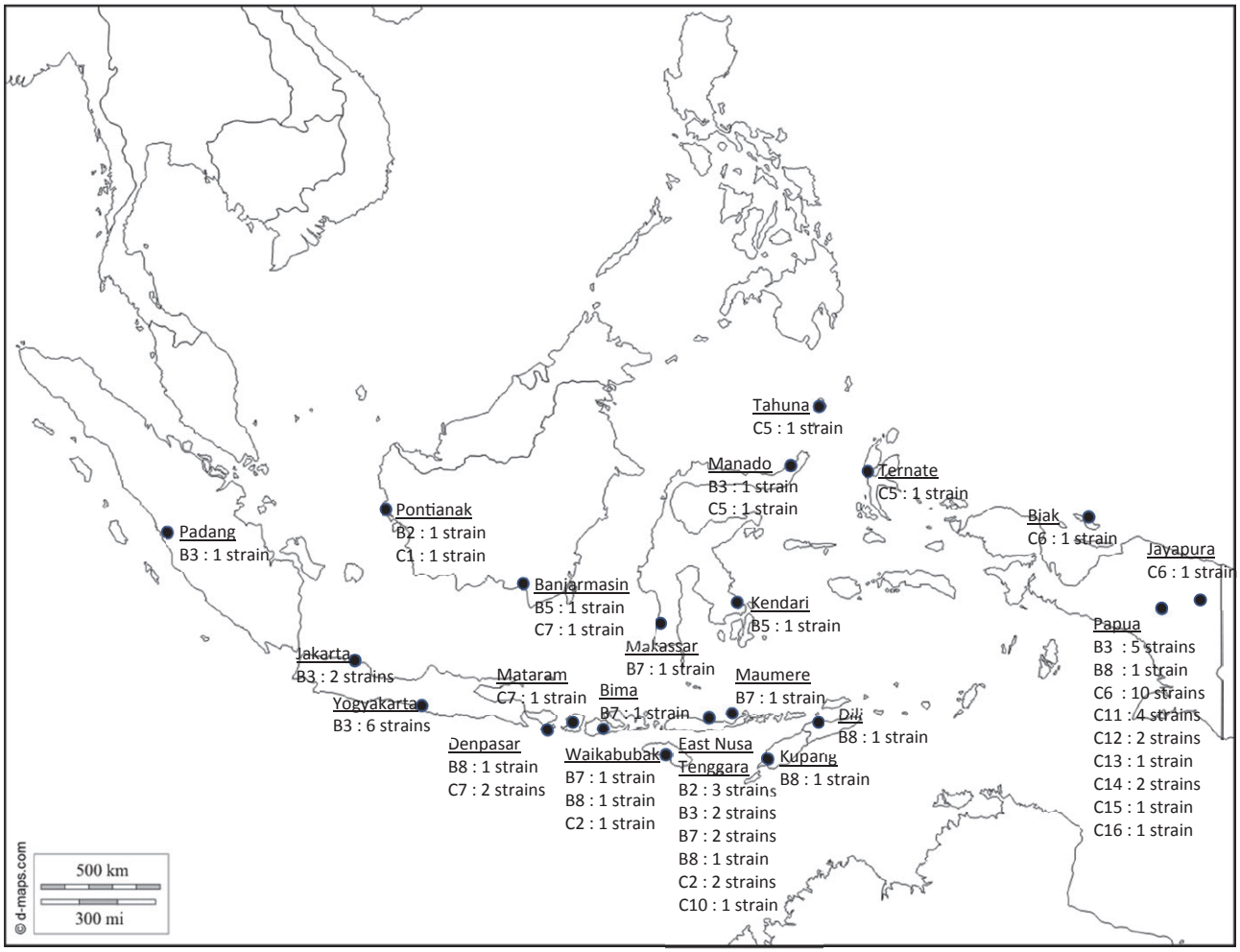

Fig. 1. Map of the Indonesian archipelago showing distribution of HBV genotypes retrieved from GenBank for this study. Our study used 96 full genome HBV that were scattered in eastern region to western region. Seventy of full genome HBV strains were shown in 21 regions of Indonesia as described in the map. While, twenty six of full genome HBV strains were not specifically identified for their location.

Fig. 2. Phylogenetic tree analysis of the complete genome sequences of 96 strains retrieved from GenBank. Fifty four representative HBV genotype B strains (B2–B3, B5, and B7–B9) and 42 HBV genotype C strains (C1–C7, C10–C11, and C13–C16) were analyzed and compared with 17 reference HBV strains of different genotypes (A–H). The strains and reference sequences are indicated by their subgenotypes, accession numbers, and region of origin. The bootstrap values are shown at the main branches. The length of the horizontal bar indicates the number of nucleotide substitutions per site.

1) were detected in 5/54 (9.3%) genotype B strains and in 1/42 (2.4%) genotype C strains. Secondary or compensatory mutations (category 2) were found only in 2/54 (3.7%) genotype B strains. The distributions of categories 1 and 2 were not significantly different between genotypes B and C.

Among putative NAr mutations (category 3), mutations rtL91I and rtY221F significantly correlated with genotype C ($P \leq 0.001$). Pretreatment mutations (category 4) were common in both genotypes. These mutations, especially rtM129L, rtD134N, rtM145L, and rtE263H/D/Q, were detected at significantly higher frequencies in genotype C strains than in genotype B strains ($P \leq 0.001$). Only mutation rtN226H/T was found to be significantly more frequent in genotype B strains than in genotype C strains ($P \leq 0.001$).

In this study, mutations that did not fit categories 1–4 were classified into the fifth category. Mutations rtR138W, rtY141F, and rtV142I, for example, were defined as novel RT mutations related to potential HBsAg escape mutations (category 5) in the surface gene e.g., sQ129H, sM133L, and sM133I, respectively. These mutations were detected only in genotype B strains.

Mutation rates in different RT regions: Mutations belonging to categories 3 and 4 (Table 2) were frequently observed in both genotypes. These mutations were scattered across the 3 regions of the RT gene. Analyses of the RT regions revealed that the abundance and frequency of mutations were the greatest in the A–B interdomain region. This region contains many mutable sites related to NA resistance, which are included in categories 3, 4, and 5. The RT domain was found to contain fewer mutable sites, such as rt91, rt233, rt248, rt256, rt263, and rt267. Meanwhile, only 2 mutable sites (rt221 and rt226) were detected in the non-A–B interdomain (Table 2). Analysis of the mutable site distribution revealed that the A–B interdomain has greater genetic variability than do the other regions, even though it is the smallest region of RT. The rate of mutations in the RT domain was not significantly different between genotypes B and C ($P = 0.059$). On the other hand, the mutation rates in the A–B and non-A–B interdomains were significantly higher in genotype C strains than in genotype B strains ($P < 0.001$; Table 3).

Overlapping region of the RT and S genes: Some mutations may occur in both RT and S genes as a consequence of the overlap between these genes. In the A–B interdomain, most of the NAr mutations characterized by high frequency were not accompanied by aa changes in the S protein. For example, mutations at sites rt129, rt134, rt139, and rt145 in the A–B interdomain did not result in aa changes at positions s121, s126, s131, and s137 in the S protein. Nonetheless, some mutations in the A–B interdomain resulted in concomitant changes in the RT and S genes that were associated with escape mutations. These mutations included rtT128I/sP120S, rtR138W/sQ129H, rtY141F/sM133L, rtV142I/sM133I, and rtW153Q/sG145R. Indeed, these mutations were rare, with frequencies of 1.0% (1/96), 3.1% (3/96), 2.1% (2/96), 1.0% (1/96), and 1.0% (1/96), respectively (Fig. 3). Meanwhile, high-frequency NAr mutations in the non-A–B interdomain were usually accompanied by aa changes in the S protein and included rtH55R/sT47M and rtY221F/sL213I (Fig. 4).

Several aa mutations in the S protein were frequently detected and affected positions s2, s17, s23, s43, s122, s177, and s207 (Fig. 4). Nevertheless, these mutations were silent and not accompanied by RT mutations at positions rt10, rt25, rt31, rt51, rt130, rt185, and rt215, respectively (data not shown).

DISCUSSION

Mutations in the HBV genome that increase heterogeneity of the viral population in an infected person occur naturally because HBV RT lacks proofreading capacity and because the mutation rate is high, with up to 10^{-5} sub-

Table 2. Prevalence of RT mutations identified in treatment-naïve Indonesian HBV carriers

No.	Mutation category	Type	Relationship with therapy	Genotype B (n = 54)[1]		Genotype C (n = 42)[1]		P-value[2]
				% Mutation	GenBank accession no.	% Mutation	GenBank accession no.	
1	Primary drug resistance mutations[3]	A194T	ADV, TNF	7.4	GQ358144, GQ358145, GQ358146, GQ358147	0.0	–	0.073
		M204V/R	LMV	1.9	EF473974	2.4	AB554025	0.858
2	Secondary/ compensatory mutations[3]	L80V	LMV	1.9	EF473974	0.0	–	0.378
		L180W/M	LMV, ETV, LdT	3.7	AB713530, EF473974	0.0	–	0.210
3	Putative NAr mutations[4]	L91I	LMV	0.0	–	19.0	AB540583, AB540584, AB540585, AB554014, AP011098, AP01109, AP011100, AP011101	0.001*
		T128I	LMV	1.9	GQ358140	0.0	–	0.378
		R153Q	LMV	0.0	–	2.4	AB554025	0.257
		Y221F	ADV	0.0	–	40.5	AB540583, AB540584, AB540585, AB644283, AB644284, GQ358153, GQ358154, GQ358158, AP011097, AP011098, AP011099, AP011100, AP011101, AP011104, AP011105, AP011106, AP011107	< 0.001*
		I233V	ADV	0.0	–	2.4	AP011100	0.257
		N248H	ADV	7.4	AP011089, AP011096, GQ358145, AB493833	4.8	AB644287, AB493842	0.478
		S256G	LMV	0.0	–	2.4	AB554018	0.257
4	Pre treatment mutations[4]	T39A	Found before therapy	13.0	AP011090, AP011091, EF473976, GQ358137, GQ358138, GQ358139, GQ358141	2.4	AB540584	0.064
		M129L		1.9	AB493834	26.2	AB540583, AB540585, AB554015, AB554019, AB554021, AB554022, GQ358155, GQ358156, AP011102, AP011103, AB554014	< 0.001*
		D134N		11.1	AP011084, EF473974, EF473975, EF473977, GQ35814, 2GQ358143	40.5	AB554015, AB554019, AB554020, AB554021, AB554022, AB644281, GQ358155, GQ358156, AB554014, AP011102, AP011103, AB493837, AB493838, AB493839, AB493840, AB493842, AB493847	0.001*
		N139K		3.7	AB713529, AB713530	11.9	AB540584, AB644286, AP0011099, AP011100, AP011101	0.127
		M145L		3.7	AB713528, AP011092	40.5	AB540583, AB540584, AB540585, AB644283, AB644284, GQ358153, GQ358154, GQ358158, AB644287, AP011097, AP011098, AP011099, AP011100, AP011101, AP011102, AP011108, AB493839	< 0.001*
		N226H/T		33.3	AB554017, AB713527, AB713528, AB713531, AB713532, AP011088, AP011090, AP011091, AP011092, EF473976, GQ358137, GQ358138, GQ358139, GQ358140, GQ358141, GQ358142, GQ358143, AB540582	2.4	GQ358158	< 0.001*
		E263H/D/Q		1.9	GQ3558139	31.0	AB554015, AB560662, AB644281, GQ358155, GQ358156, AP011099, AP011100, AP011101, AP011104, AP011105, AP011106, AP011107, AB493837	< 0.001*
		L267Q/F/S/V		25.9	AP011084, AP011088, AP011096, EF473973, EF473974, EF473975, EF473977, GQ358142, GQ358143, GQ358148, GQ358149, GQ358150, GQ358151, GQ358152	31.0	AB540583, AB540585, AB554020, AB644281, GQ358153, AP011097, AP011098, AP011103, AP011104, AP011105, AP011106, AP011107, AP011108	0.589
5	Novel mutations related to potential escape mutations[5]	R138W	–	3.7	AB7713527, AB713532	0.0	–	0.210
		Y141F		3.7	EF473974, AB033555	0.0	–	0.210
		V142I		1.9	AP011086	0.0	–	0.378

[1]: Using strain EF473976 as consensus for strain with genotype B and strain AB540583 for strain with genotype C to determine amino acid changes.
[2]: Statistical significance was calculated using two independent samples t-test for distributed normally data and Mann-Whitney U test for non-normally distributed data. Significant values are indicated with asterisks.
[3]: Well known NA resistance mutations with phenotypic data.
[4]: The functional relevance of several mutations has not been clarified.
[5]: New mutations found in this study and related HBsAg escape mutations.
ADV, adefovir dipivoxil; ETV, entecavir; Ldt, telbivudine; LMV, lamivudine; TNF, tenofovir.

stitutions/base/cycle during HBV replication. Several studies have shown that naturally occurring mutations associated with NAr are present in some patients before initiating of treatment (29). Nevertheless, there are no published reports describing the RT mutational pattern in HBV strains obtained from Indonesian populations. The present study is the first to compare the prevalence of naturally occurring NA resistance mutations between HBV genotype B strains and genotype C strains isolated from treatment-naïve Indonesian HBV carriers and reported previously in GenBank.

The whole genomes of 96 HBV strains retrieved from

Table 3. Prevalence of mutations in specific RT regions

RT region	No. of sites	Mean mutation rate (%)[1)]		Mean difference (95% CI)	*P*-value[2)]
		Genotype B (n = 54)	Genotype C (n = 42)		
RT domain[3)]	115	1.06 ± 0.89	1.45 ± 1.05	0.39 (0.01–0.78)	0.059
A–B interdomain[4)]	72	1.08 ± 1.52	3.51 ± 2.53	2.43 (1.59–3.25)	< 0.001*
Non-A–B interdomain[5)]	157	1.16 ± 0.88	2.58 ± 1.51	1.42 (0.93–1.19)	< 0.001*

1): The mutation rate/domain = number of mutations in each domain/total number of sites in that domain. For example, in 1 strain with 2 mutations in the RT domain (115 sites), the mutation rate = 2/115 (1.7%).
2): Mann-Whitney *U* test. Significant values are indicated with asterisks.
3): Mutation sites were assessed in RT domains, domain F (rt33–rt47), A (rt70–rt91), B (rt164–rt190), C (rt199–rt211), D (rt229–rt241), and E (rt246–rt270).
4): Mutation sites in the A–B interdomain include rt92–rt163.
5): Mutation sites in the non-A–B interdomain include spacer–F (rt1–rt32), F–A (rt48–rt69), B–C (rt191–rt198), C–D (rt212–rt228), D–E (rt242–rt245), and E–RNA H (rt271–rt344).
CI, confidence interval.

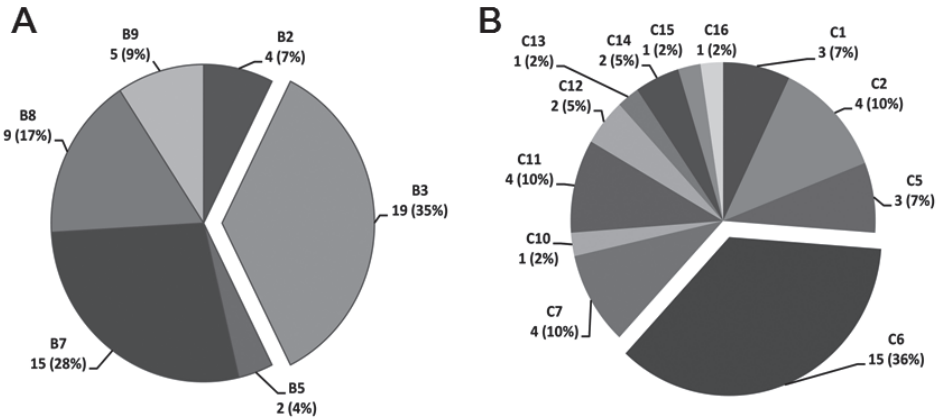

Fig. 3. Percentages of subgenotype distribution in the genotype B (A) and C (B) strains analyzed in this study. Six and 12 subgenotypes were found in genotypes B and C, respectively.

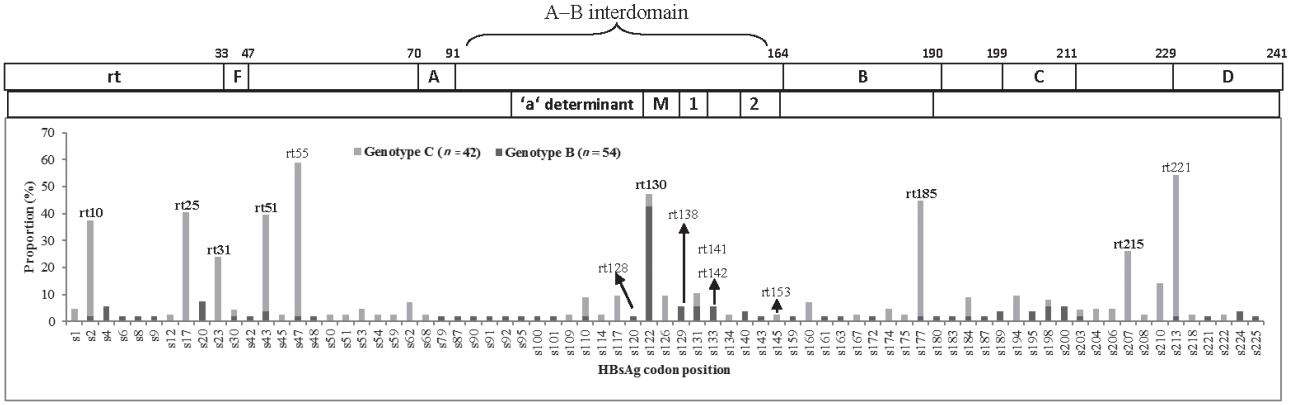

Fig. 4. Analysis of the concomitant amino acid (aa) changes in the RT and S proteins in genotype B and C strains (n = 96). Location of the overlapping S gene in the RT gene. Typical aa changes, including rtH55R/sT47M and rtF221Y/sL213I, were associated with aa changes in the RT and S proteins. Some of the mutations in the A–B interdomain are accompanied by aa changes associated with escape mutations, for instance, rtT128I/sP120S, rtR138W/sQ129H, rtY141F/sM133L, rtV142I/sM133I, and rtW153Q/sG145R. Although several aa substitutions in the S gene were frequent (e.g. rt10/s2, rt25/s17, rt31/s23, rt51/s43, rt130/s122, rt185/s177, and rt215/s207), they were not accompanied with changes in the RT region marked by bold letter. rt, reverse transcriptase; functional domains of RT (F, A, B, C, and D); M, mini-loop; 1, loop 1; 2, loop 2.

GenBank correspond to treatment-naïve Indonesian HBV carriers and represent some regions of Indonesia. Naturally occurring HBV strains bearing primary drug resistance mutations are very rare in the absence of prior treatment. In this study, primary drug resistance mutation rtA194T was more frequent than rtM204V (4.2% [4/96] vs. 2.1% [2/96]). It was recently revealed that rtA194T confers decreased susceptibility to tenofovir (30). In contrast, the influence of rtA194T according to other investigators is not completely consistent. One study has shown that HBV with mutation rtA194T remains susceptible to tenofovir in vitro (31). The presence of mutations at position rtM204 leads to lamivudine and entecavir resistance in HBV-infected patients (32). Secondary/compensatory mutations were detected only in 2/54 (3.7%) genotype B strains. One strain had mutation rtL80V together with rtM204V. Moreover, the primary and secondary mutations are located in the RT domain. These RT domains are responsible for drug binding. The primary and secondary mutations have been shown to ensure that an antiviral drug would not work effectively because the mutations is associated with a change in the structure of the drug-binding region (33).

Prolonged NA therapy favors the selection of putative NAr mutations that are associated with NAr or replication compensation. Nevertheless, the functional relevance of these mutations has not been fully clarified in vitro. These mutations were found to be evenly distributed throughout the RT region. Of note, this study showed that putative NAr mutations were already present in viruses of treatment-naïve patients, for instance rtL91I, rtT128I, rtR153Q, rtY221F, rtI233V, rtN248H, and rtS256G. Although these mutations may not be wholly responsible for the nonresponse to NA therapy, they may decrease HBV susceptibility to antiviral drugs during treatment. On the other hand, in vitro and follow-up studies are needed to resolve this issue. Earlier studies have provided evidence that supports the influence of putative NAr mutations. Mutations at positions rtL91 and rtI91 in domain A were more frequent in genotypes B and C, respectively. The combination of mutations at rtL91 and rtC256 was frequently detected in patients with extended treatment failure. It was suggested that the detection of this combination of mutations before lamivudine therapy is related to the early outbreak of NAr mutations (11). In the present study, only 2 strains had mutations at both positions rtL91 and rtC256. Other studies have shown that the rtT128N and rtW153Q mutations in the A–B interdomain partially restore the replication capacity of lamivudine-resistant HBV (8). Our results indicate that mutations at positions rtY221 and rtF221 in the non-A–B interdomain are prevalent in genotypes B and C, respectively. The mutation at position rtY221 was identified as a new mutation and may be relevant to the development and evolution of resistance to oral antiviral therapy. Its frequency is higher in HBV genotype A patients being treated with adefovir dipivoxil than in treatment-naïve patients (34). Nonetheless, the relation between these mutations in genotypes B or C and antiviral resistance are still unknown. The rtI233V mutation in domain D has been implicated in the resistance to adefovir dipivoxil but not to tenofovir (35). It also contributes to compensation for replication defects in multidrug-resistant mutants (6). In contrast, the role of the rtI233V mutation in adefovir dipivoxil resistance is still controversial (36). An in vitro study has revealed that the appearance of rtN248H increases resistance to adefovir dipivoxil 5.71-fold, but the strain is still susceptible to lamivudine, telbivudine, entecavir, and tenofovir (37). The rtS256G mutation in domain E is associated with entecavir treatment (9) and has been detected in genotype B but not in genotype C strains in one study (11). By contrast, in the present study, this mutation was found only in 1 genotype C strain. The number of putative NAr mutations was higher in genotype C strains than in genotype B strains. The clinical significance of putative NAr mutations in both genotypes requires further verification.

The other commonly found mutations include rtT39A, rtM129L, rtD134N, rtN139K, rtM145L, rtN226H/T, rtE263H/D/Q, and rtL267Q/F/S/V, which were classified as pretreatment mutations. Their relation to antiviral resistance should be evaluated in future studies. One research group concluded that the rt106 and rt134 mutations in RT may be associated with necroinflammation, an immune response, and progression to cirrhosis in treatment-naïve patients, and the study indicated that HBV strains with mutations at position rt106 or rt134 might facilitate the progression of liver disease (38). Although the mutation at position rt106 was not detected in this study, mutation of rt134 was found in 40.5% of genotype C strains. This result indicates that a person infected with HBV genotype C probably has a severer disease in comparison with genotype B. This notion requires confirmation by comparing the prevalence of this mutation among cases of advanced and non-advanced liver disease in Indonesian patients.

Mutations rtR138W, rtY141F, and rtV142I were classified into a new category and may occur after emergence of possible escape mutations in the S gene (sQ129H, sM133I, and sF134L, respectively; Table 2). Therefore, mutations in the RT region may predict decreasing antigenicity of HBsAg.

The HBV RT region consists of 6 functional domains (F, A, B, C, D, and E) and 5 interdomains (F–A, A–B, B–C, C–D, and D–E) (39). In line with other studies, the RT domain was found to have the lowest mutation rate. This is understandable because changes in the RT domain may affect HBV replication in the absence of antiviral drugs (11). The A–B interdomain showed the highest mutation rate, suggesting that it is a highly mutagenic region. A mutation in the A–B interdomain may not directly affect RT function or confer antiviral resistance because this region is structurally distant from the functional domain. Nonetheless, a part of the A–B interdomain overlaps the "a" determinant region (s124–147) and the major hydrophilic region (s99–169) within HBsAg. Thus, mutations in these regions may alter the antigenic loops of HBsAg and reduce the binding affinity of neutralizing antibodies. Therefore, mutations in the "a" determinant region or major hydrophilic region may result in vaccine/HBIg escape (6).

Concomitant substitutions in the RT and S genes that contribute to viral immune escape were found in the A–B interdomain and included rtT128I/sP120S, rtR138W/sQ129H, rtY141F/sM133L, rtV142I/sM133I, and rtW153Q/sG145R. These mutations have been reported

elsewhere (6,8,10). Although the RT and S genes overlap, aa changes in the S protein were not necessarily caused by RT mutations. Conversely, the aa changes caused by mutations in the S gene might also have been selected by immune pressure from the host without an aa change in RT. This study showed that mutations in the S gene causing aa changes are more frequent in genotype C than in genotype B strains.

In this study, we systematically investigated the RT mutations and their effects on the aa sequence of the S protein in treatment-naïve Indonesian patients carrying HBV of genotype B or C. Nevertheless, the potential role of HBV genotypes in the emergence of NA resistance is still unclear. Cohort studies on patients with HBV of genotype B or C undergoing NA therapy are needed to address the relevance of mutations that might reduce susceptibility to NA and play a role in antiviral-drug resistance. Moreover, several naturally occurring RT mutations in the viruses of treatment-naïve patients may be related to reduced effectiveness of drugs and viral immune escape. A better understanding of the characteristics of HBV RT and S sequences is crucial for preventing and controlling HBV infection in Indonesia.

Acknowledgments The authors would like to thank Dewi Ismimasitoh (Data Management and Analysis Clinical Epidemiology and Biostatistics Unit, Medical Faculty of Gadjah Mada University/Dr. Sardjito Hospital, Yogyakarta, Indonesia) for her help to review methods of biomedical statistics used. This work was partly supported by Grant-in-Aid for Scientific Research (B; Grant Number: 16H05826).

Conflict of interest None to declare.

REFERENCES

1. Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. J Hepatol. 2006;44:422-31.
2. Pastor R, Habersetzer F, Fafi-Kremer S, et al. Hepatitis B virus mutations potentially conferring adefovir/tenofovir resistance in treatment-naive patients. World J Gastroenterol. 2009;15:753-5.
3. Solmone M, Vincenti D, Prosperi MC, et al. Use of massively parallel ultradeep pyrosequencing to characterize the genetic diversity of hepatitis B virus in drug-resistant and drug-naive patients and to detect minor variants in reverse transcriptase and hepatitis B S antigen. J Virol. 2009;83:1718-26.
4. Panigrahi R, Biswas A, De BK, et al. Characterization of antiviral resistance mutations among the Eastern Indian hepatitis B virus infected population. Virol J. 2013;10:56.
5. Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. J Hepatol. 2006;44:593-606.
6. Locarnini S. Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a consequence of treatment failure. Hepatol Int. 2008;2:147-51.
7. Lai CL, Leung N, Teo EK, et al. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. Gastroenterology. 2005;129:528-36.
8. Torresi J, Earnest-Silveira L, Civitico G, et al. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the "fingers" subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology. 2002;299:88-99.
9. Colonno RJ, Rose R, Baldick CJ, et al. Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. Hepatology. 2006;44:1656-65.
10. Sheldon J, Soriano V. Hepatitis B virus escape mutants induced by antiviral therapy. J Antimicrob Chemother. 2008;61:766-8.
11. Liu BM, Li T, Xu J, et al. Characterization of potential antiviral resistance mutations in hepatitis B virus reverse transcriptase sequences in treatment-naïve Chinese patients. Antiviral Res. 2010;85:512-9.
12. Ogura Y, Kurosaki M, Asahina Y, et al. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. J Infect Dis. 1999;180:1444-51.
13. Pollicino T, Isqro G, Di Stefano R, et al. Variability of reverse transcriptase and overlapping S gene in hepatitis B virus isolates from untreated and lamivudine-resistant chronic hepatitis B patients. Antivir Ther. 2009;14:649-54.
14. Utsumi T, Yano Y, Lusida MI, et al. Serologic and molecular characteristics of hepatitis B virus among school children in East Java, Indonesia. Am J Trop Med Hyg. 2010;83:189-93.
15. Mulyanto, Depamede SN, Surayah K, et al. A nationwide molecular epidemiological study on hepatitis B virus in Indonesia: identification of two novel subgenotypes, B8 and C7. Arch Virol. 2009;154:1047-59.
16. Thedja MD, Muljono DH, Nurainy N, et al. Ethnogeographical structure of hepatitis B virus genotype distribution in Indonesia and discovery of a new subgenotype, B9. Arch Virol. 2011;156:855-68.
17. Utama A, Purwantomo S, Siburian MD, et al. Hepatitis B virus subgenotypes and basal core promoter mutations in Indonesia. World J Gastroenterol. 2009;15:4028-36.
18. Utama A, Siburian MD, Fanany I, et al. Low prevalence of hepatitis B virus pre-S deletion mutation in Indonesia. J Med Virol. 2011;83:1717-26.
19. Utama A, Siburian MD, Fanany I, et al. Hepatitis B virus pre-S2 start codon mutations in Indonesian liver disease patients. World J Gastroenterol. 2012;18:5418-26.
20. Sastrosoewignjo RI. The complete nucleotide sequence of an HBV DNA clone of subtype *adw* (pRTB299) from Indonesia. ICMR Ann. 1985;5:39-50.
21. Sastrosoewignjo RI. The complete nucleotide sequence of HBV DNA clone of subtype *adw* (pMND122) from Menado in Sulawesi Island, Indonesia. ICMR Ann. 1987;7:51-60.
22. Nurainy N, Nuljono DH, Sudoyo H, et al. Genetic study of hepatitis B virus in Indonesia reveals a new subgenotype of genotype B in east Nusa Tenggara. Arch Virol. 2008;153:1057-65.
23. Utsumi T, Lusida MI, Yano Y, et al. Complete genome sequence and phylogenetic relatedness of hepatitis B virus isolates in Papua, Indonesia. J Clin Microbiol. 2009;47:1842-7.
24. Mulyanto, Depamede SN, Surayah K, et al. Identification and characterization of novel hepatitis B virus subgenotype C10 in Nusa Tenggara, Indonesia. Arch Virol. 2010;155:705-15.
25. Juniastuti, Utsumi T, Nugrahaputra VE, et al. Another novel subgenotype of hepatitis B virus genotype C from papuans of Highland origin. J Med Virol. 2011;83:225-34.
26. Mulyanto, Depamede SN, Wahyono A, et al. Analysis of the full-length genomes of novel hepatitis B virus subgenotypes C11 and C12 in Papua, Indonesia. J Med Virol. 2011;83:54-64.
27. Mulyanto, Pancawardani P, Depamede SN, et al. Identification of four novel subgenotypes (C13–C16) and two inter-genotypic recombinants (C12/G and C13/B3) of hepatitis B virus in Papua province, Indonesia. Virus Res. 2012;163:129-40.
28. Rinonce HT, Yano Y, Utsumi T, et al. Hepatitis B and C virus infection among hemodialysis patients in Yogyakarta, Indonesia: prevalence and molecular evidence for nosocomial transmission. J Med Virol. 2013;85:1348-61.
29. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol. 1995;13:29-60.
30. Dupouey J, Gerolami R, Solas C, et al. Hepatitis B virus variant with the a194t substitution within reverse transcriptase before and under adefovir and tenofovir therapy. Clin Res Hepatol Gastroenterol. 2012;36:e26-8.
31. Zhu Y, Curtis M, Borroto-Esoda K. The YMDD and rtA194T mutations result in decreased replication capacity in wild-type HBV as well as in HBV with precore and basal core promoter mutations. Antivir Chem Chemother. 2011;22:13-22.
32. Mukaide M, Tanaka Y, Shin-I T, et al. Mechanism of entecavir resistance of hepatitis B virus with viral breakthrough as determined by long-term clinical assessment and molecular docking simulation. Antimicrob Agents Chemother. 2010;54:882-9.
33. Michailidis E, Kirby KA, Hachiya A, et al. Antiviral therapies: focus on hepatitis B reverse transcriptase. Int J Biochem Cell Biol. 2012;44:1060-71.
34. Mirandola S, Sebastiani G, Rossi C, et al. Genotype-specific mutations in the polymerase gene of hepatitis B virus potentially associated with resistance to oral antiviral therapy. Antiviral Res. 2012;96:422-9.

35. Schildgen O, Sirma H, Funk A, et al. Variant of hepatitis B virus with primary resistance to adefovir. N Engl J Med. 2006;354:1807-12.
36. Tan J, Degertekin B, Wong SN, et al. Tenofovir monotherapy is effective in hepatitis B patients with antiviral treatment failure to adefovir in the absence of adefovir-resistant mutations. J Hepatol. 2008;48:391-8.
37. Qin B, Budeus B, Cao L, et al. The amino acid substitutions rtP177G and rtF249A in the reverse transcriptase domain of hepatitis B virus polymerase reduce the susceptibility to tenofovir. Antiviral Res. 2013;97:93-100.
38. Zheng J, Zeng Z, Zhang D, et al. Prevalence and significance of hepatitis B reverse transcriptase mutants in different disease stages of untreated patients. Liver Int. 2012;32:1535-42.
39. Stuyver LJ, Locarnini SA, Lok A, et al. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology. 2001;33:751-7.