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A Deep-Sequencing Method Detects Drug-Resistant Mutations in the Hepatitis B Virus in Indonesians

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Key Words

Hepatitis B virus · Deep sequencing · Nucleos(t)ide analogue · Indonesia

Abstract

Objective: The long-term administration of a nucleos(t)ide analogue (NA) for the treatment of chronic hepatitis B may encourage the emergence of viral mutations associated with drug resistance. Minor populations of viruses may exist before treatment, but are difficult to detect because of technological limitations. Identifying minor viral quasispecies should be useful in the clinical management of hepatitis B virus (HBV) infection. ***Methods:*** Six treatment-naïve Indonesian patients with chronic HBV infection participated in this study. The polymerase region of the HBV genome, including regions with known drug-resistant mutations, was subjected to capillary sequencing and MiSeq sequencing (Illumina). Mutations were analyzed with Genomics Workbench software version 6.0.1 (CLC bio). ***Results:*** The mean mapping reads for the six samples was 745,654, and the mean number of amplified fragments ranged from 17,926 to 25,336 DNA reads. Several known drug-resistant mutations in the reverse transcriptase region were identified in all patients, although the frequencies were low (0.12–1.06%). The proportions of the total number of reads containing mutations I169L/M, S202R, M204I/L or N236S were >1.0%. ***Conclusion:*** Several known NA-resistant mutations were detected in treatment-naïve patients in Indonesia using deep sequencing. Careful management of such patients is essential to prevent drug-resistant mutations from spreading to other patients.

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Introduction

Approximately 350 million people are infected with hepatitis B virus (HBV) worldwide, including asymptomatic carriers, making HBV infection a major global health problem. Chronic HBV infection often progresses to liver cirrhosis and hepatocellular carcinoma, and around 1 million deaths per year are thought to be related to HBV infection [1]. The prevalence of HBV infection varies considerably between countries. In Indonesia, it is reported that 4.0–20.3% of the population are infected with HBV, and Indonesia is considered to be a moderate-to-high endemic region [2].

The purpose of chronic hepatitis B (CHB) therapy is the sustained suppression of HBV replication and the remission of liver disease. The nucleos(t)ide analogues (NAs) used to treat CHB include lamivudine (LAM), telbivudine (L-deoxythymidine; LdT), entecavir (ETV), adefovir dipivoxil (ADV), tenofovir and emtricitabine. NAs mainly suppress viral proliferation by inhibiting HBV DNA polymerase activity. They are generally well tolerated and are easy to administer once daily. They also reduce liver damage, improve liver function and inhibit hepatocarcinogenesis in chronically HBV-infected patients with fewer adverse effects than conventional interferon therapy [3, 4]. However, the emergence of drug-resistant mutations during long-term treatment is a major limitation of these drugs. Drug-resistant mutations were found in approximately 70% of patients treated with LAM for up to 5 years and in 3% of patients treated with ADV for up to 3 years. Resistance to ETV was reported in 1.1% of patients treated for up to 4 years [5–7]. Numerous cases of severe reactivation, viral relapse and exacerbation of hepatitis after discontinuation of NA therapy have also been reported [8].

The HBV genome is a partially double-stranded circular DNA molecule of approximately 3,200 bp, consisting of four overlapping open reading frames that encode the surface protein, the core protein, a polymerase and region X, which encodes a multifunctional nonstructural protein. The polymerase region is an important target for NAs, and mutations in this region are sometimes associated with drug resistance. HBV replication is very fast, with up to 10^{11} viral particles released per day. This high replication rate, coupled to the absence of a proofreading mechanism in the HBV polymerase, contributes to the appearance of mutations [9, 10]. Mutations in the reverse transcriptase (RT) region sometimes induce viral breakthrough. These mutations include drug-induced mutations after NA treatment, and natural mutations or viral quasispecies present prior to treatment [3, 5].

However, it is currently very difficult to detect viruses with drug-resistant mutations that are present in very low copy numbers because of technological limitations. Next-generation sequencing methods are used to sequence thousands to millions of sequences concurrently, allowing us to detect any preexisting mutations before antiviral therapy is commenced. Identifying minor viral quasispecies and low-frequency NA-resistant mutations in treatment-naïve patients should allow us to select the most appropriate NA regimen and to predict the future emergence of mutations, offering significant benefits in the management of patients with HBV infection. Although some studies have used next-generation sequencing methods to detect NA-resistant mutations, most of the patients had HBV genotype C [11, 12].

In this study, we sought to identify quasispecies in the RT region of the HBV genome and detect possible drug-resistant mutations in Indonesian treatment-naïve patients with CHB, using a next-generation sequencer. The results are expected to be useful for evaluating the risk of emerging drug-resistant mutations and selecting the most appropriate antiviral drug for patients with CHB.

Materials and Methods

Subjects

To detect preexisting mutations associated with NA resistance in Indonesian patients, we obtained serum samples at the inpatient and outpatient units of the Gastroenterology and Hepatology Department of Internal Medicine, Dr. Sardjito Hospital, Yogyakarta, Indonesia, from 6 treatment-naïve chronic HBV carriers. The patients were 22–77 years old (median 38 years). Three of the patients were asymptomatic carriers and the other 3 had advanced liver disease: hepatocellular carcinoma in 2 and liver cirrhosis in 1. Alanine aminotransferase and aspartate aminotransferase levels were measured immediately after blood collection. Informed consent was obtained from all of the patients. The study was approved by the Medical and Health Ethics Committees of Gadjah Mada University and Kobe University (Japan).

DNA Extraction and Measurement of the HBV Viral Load

HBV DNA was extracted from 200 µl of sera using a Qiagen DNA Blood Mini Kit, according to the manufacturer's instructions (Qiagen, Tokyo, Japan). The HBV viral load was measured with real-time polymerase chain reaction (PCR) on an ABI Prism 7700 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA), using a previously described specific primer and probe set [13].

Direct PCR Sequencing and Genotype Determination

The RT region was amplified and confirmed with a PCR assay using specific primers [14]. After the first round of PCR with the HB8F and HB6R primers, a nested PCR was performed with the HB2F and HB3R primers. The amplified products (694 bp) ob-

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Table 1. Clinical characteristics of patients and results of next-generation sequencing

	Patient ID					
	B55	B77	B149	B13	B126	B143
Age, years	40	22	28	77	55	36
Sex	male	male	female	male	female	male
Diagnosis	AC	AC	AC	HCC	HCC	cirrhosis
Viral load, log copies/ml	7.7	7.7	2.7	<2.6	<2.6	3.9
AST, IU/l	74	152	31	74	31	115
γ-GTP, IU/l	40	27	17	8	19	30
Genotype	B3	B3	B3	B3	B3	B3
Total reads	1,781,062	1,327,708	1,638,654	1,260,250	1,298,306	1,413,446
Mapping reads	914,396	679,886	837,533	648,284	667,297	726,531
Mean coverage per nucleotide	25,336	18,191	23,372	17,926	18,519	20,589

AC = Asymptomatic carrier; HCC = hepatocellular carcinoma; AST = aspartate aminotransferase; γ-GTP = γ-glutamyl transpeptidase.

tained with the nested PCR were directly sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing Kit on an ABI Prism® 3100-*Avant* Genetic Analyzer (Applied Biosystems). The HBV genotypes were determined using a phylogenetic tree of the S region. Reference sequences were retrieved from the Japan/European Molecular Biology Laboratory, and GenBank DNA databases. Sequences were aligned using ClustalX software (www.clustal.org). Phylogenetic trees were constructed using the neighbor-joining method and bootstrap resampling was performed 1,000 times. The analyses were conducted with Molecular Evolutionary Genetics Analysis (MEGA) software [15].

PCR Products and Short-Read Sequencing

The concentration of each nested PCR product (694 bp) was measured using a Qubit dsDNA HS Assay Kit (Q32851; Invitrogen, Carlsbad, Calif., USA). A library of PCR products (<500 bp) of the viral genome (50 or 0.2 ng) was prepared using Nextera DNA Sample Prep Kit (Illumina, San Diego, Calif., USA) or Nextera XT DNA Sample Prep Kit (Illumina), according to the manufacturer's instructions. The PCR products were uniformly sheared into 500-bp fragments using these kits and the PCR product libraries were mixed with 1% 8 pM PhiX as controls, and then run on a MiSeq sequencer (Illumina) for paired-end 151-bp sequencing. The fluorescent images were analyzed using the MiSeq control software and reporter analysis (Illumina) to obtain FASTQ-formatted sequence data.

Sequence Read Mapping and Data Analysis

In accordance with the recommendation of Illumina, the sequences were used when the read quality estimated by the Q30 (quality score 30) was over 80% of the consensus sequence. After a quality check and data trimming, Genomics Workbench software version 6.0.1 (CLC bio, Aarhus, Denmark) was used to assemble the sequences. Sequence reads were mapped against the reference HBV genome (AB713528). Single-nucleotide polymorphisms were identified with probabilistic variant detection modules using the default parameters in the mapping algorithm.

The characteristics of the viral quasispecies were evaluated in terms of their genetic complexity based on the number of different sequences present in the population. To achieve this, we used the setting 'read conflicts' in Genomic Workbench. When mapping was completed, conflicts between the sequence reads were annotated on the consensus sequence. The definition of a conflict is a position at which at least one of the sequence reads encodes a different nucleotide.

Statistical Analysis

Continuous variables were compared with Student's t test or analysis of variance. $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, Ill., USA).

Results

RT Genome Alignment

The characteristics of the subjects enrolled in this study are summarized in table 1. We sequenced and analyzed part (encoding amino acids 119–306) of the RT region. Figure 1 shows the deduced protein sequence alignment of the six samples obtained with the direct-sequencing method. The capillary-sequencing method detected no drug-resistant mutations. The phylogenetic tree revealed that all samples examined in this study belonged to genotype B3 (fig. 2).

NA-Resistant Mutations Detected with Next-Generation Sequencing

The number of mapping reads in the six samples ranged from 648,284 to 914,396, and the coverage

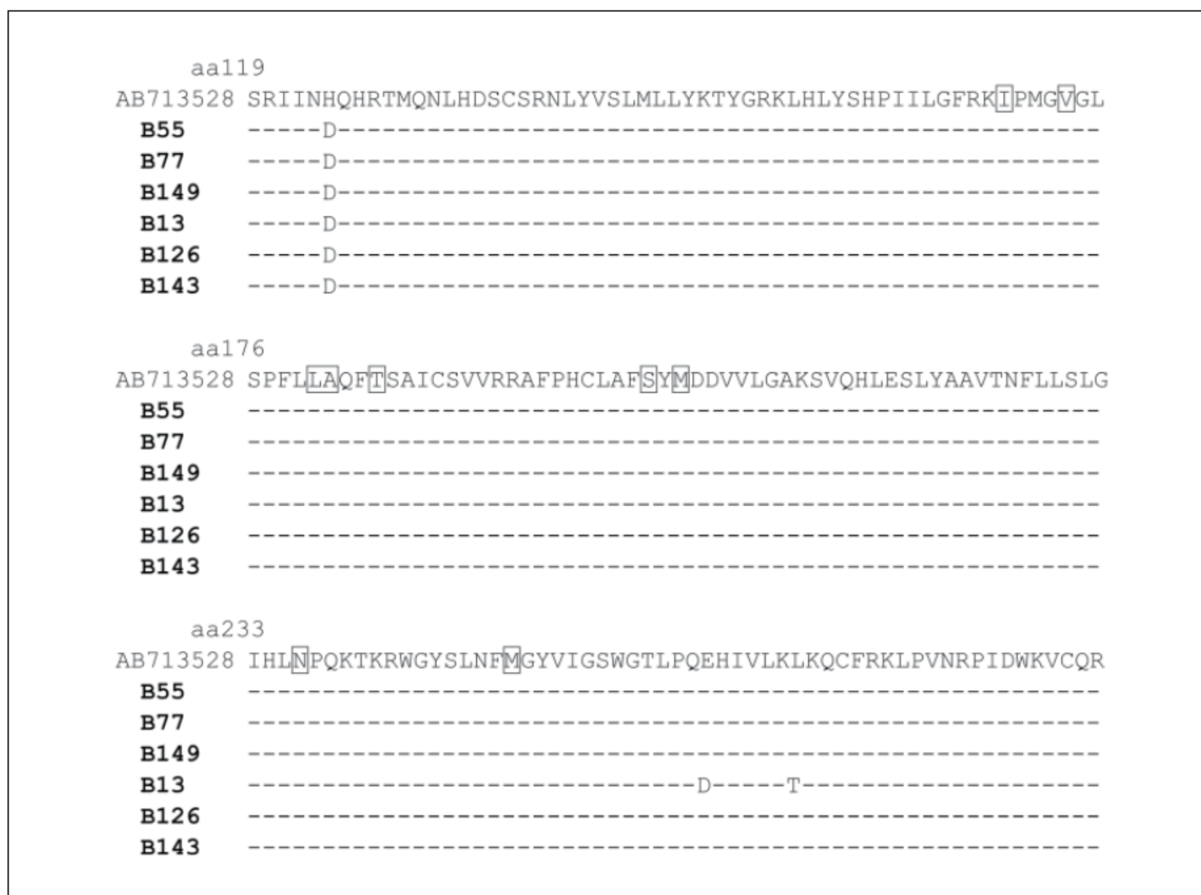

Fig. 1. Amino acid sequence alignment of the RT region of the HBV genome. The amino acid sequences were obtained by direct sequencing. The amino acid sequences obtained for the Indonesian treatment-naïve patients in this study (bold) were compared with those of other Indonesian isolates (top row). The residues within squares show the nine known positions of the NA resistance (rt169, rt173, rt180, rt181, rt184, rt202, rt204, rt236 and rt250).

ranged from 17,926 to 25,366 (table 1). Preexisting NA-resistant mutations in nine known positions of the RT region (rt169, rt173, rt180, rt181, rt184, rt202, rt204, rt236 and rt250) were detected in all 6 patients, although the frequencies of these mutations were low (0.12–1.06%). The frequencies of the amino acid substitutions M204I (conferring resistance to LAM/ETV), A181T (to LAM/ADV), A181V (to ADV) and T184A (to ETV) ranged from 0.91 to 1.06%, and were greater than those of the other amino acid substitutions. The frequencies of M204V (conferring resistance to LAM/ETV), S202G (to ETV), T184S (to ETV), N236T (to ADV) and M250V (to ETV) ranged from 0.48 to 0.69%. The frequencies of the LAM/ETV-resistant substitutions L180M and I169T ranged from 0.12 to 0.2% (table 2).

The next-generation sequencing method also revealed several other substitutions with frequencies ≥1%: I169M, I169L, V173A, S202R, M204L and N236S (table 3). These mutations have not previously been reported to be associated with NA resistance. Statistical analysis revealed that the prevalence of preexisting NA-resistant mutations was not significantly different between the chronic asymptomatic patients and the patients with advanced liver disease (table 4).

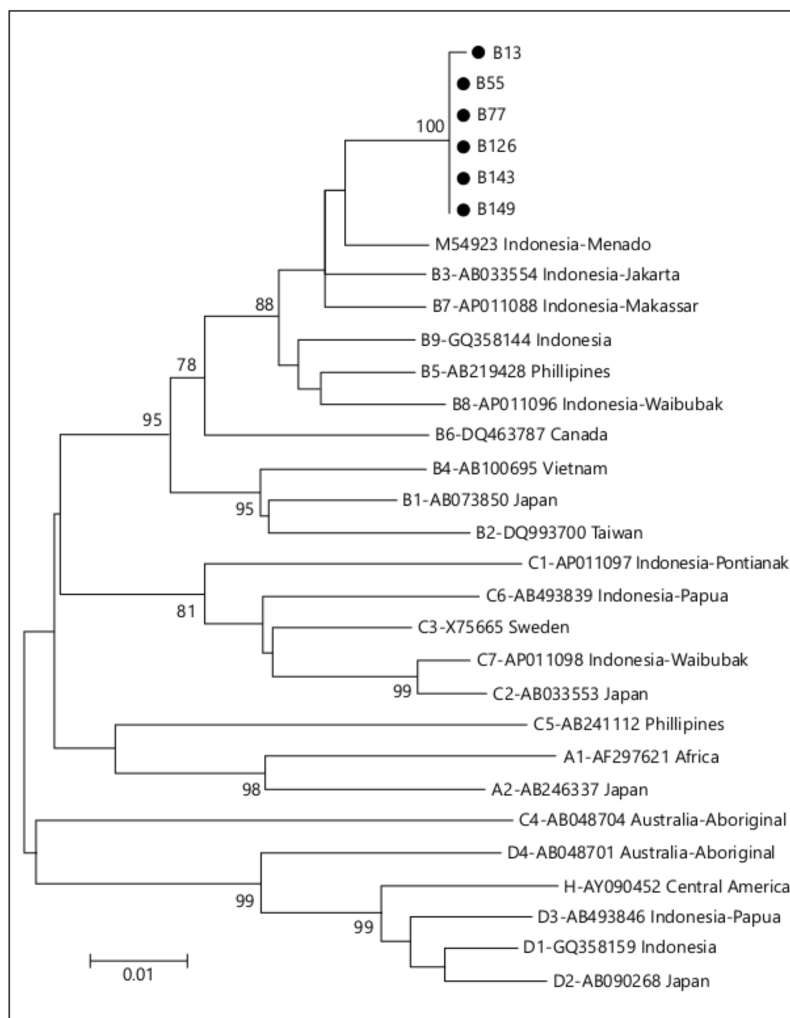

Fig. 2. Neighbor-joining phylogenetic tree for HBV strains in Indonesian treatment-naïve patients (indicated with solid circles). The phylogenetic analysis was carried out based on 694 bp of the partial S region. The bootstrap support for the consensus tree, inferred from 1,000 replicates, is indicated for each branch. The evolutionary distances were computed using the maximum composite likelihood method.

Discussion

The emergence of drug resistance is a serious problem during treatment with NA. The inhibition of HBV replication with NA may lead to the emergence of several mutations that are translated into specific amino acid changes that confer antiviral resistance [16]. Drug-resistant viruses show increased levels of HBV DNA in the liver and blood, a phenomenon known as 'virological breakthrough' or 'clinically induced hepatitis flare' [17]. The presence of genomic quasispecies may facilitate the emergence of multidrug resistance. Several studies have demonstrated the higher relative sensitivity of ultra-deep sequencing compared with conventional direct-population sequencing. Therefore, ultra-deep sequencing is useful for detecting low-frequency drug-resistant mutations that cannot be detected with standard methods [18–21]. Conventional sequencing based on the Sanger method can only detect mutations present in >20% of viral quasispecies. Although the line probe assay is another common and convenient method to detect mutations, it can only detect mutations present in >15% of viral quasispecies [20]. Cloning-based sequencing methods are also commonly used, but they usually detect less than 50% of the substitutions identified with ultra-deep sequencing [21]. The relationship between the viral dy-

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Table 2. Known NA-resistant mutations in the RT region of the HBV genome detected with next-generation sequencing

Position/ substitution	NA resistance	Patient ID						Mean ± SD
		B13	B55	B77	B126	B143	B149	
rt169/I169T	ETV	0.19	0.19	0.2	0.18	0.19	0.19	0.19±0.01
rt173/V173L	LAM	0.31	0.28	0.29	0.24	0.26	0.27	0.28±0.24
rt180/L180M	LAM/ETV	0.12	0.12	0.12	0.11	0.13	0.12	0.12±0.01
rt181/								
A181T	LAM/ADV/LdT	0.31	0.3	0.31	0.3	0.27	0.32	0.30±0.17
A181V	ADV/LdT	0.34	0.34	0.36	0.38	0.38	0.38	0.36±0.19
rt184/								
T184A	ETV	0.96	0.98	0.99	0.92	0.91	0.94	0.95±0.32
T184S	ETV	0.5	0.49	0.51	0.50	0.48	0.58	0.51±0.01
rt202/S202G	ETV	0.51	0.53	0.54	0.54	0.52	0.53	0.53±0.11
rt204/								
M204V	LAM/ETV	0.63	0.63	0.54	0.61	0.57	0.63	0.60±0.27
M204I	LAM/LdT	1.05	1.03	0.59	1.06	1.06	1.05	0.97±0.19
rt236/N236T	ADV	0.67	0.64	0.68	0.69	0.68	0.68	0.67±0.02
rt250/M250V	ETV	0.33	0.33	0.37	0.37	0.35	0.33	0.34±0.02

Values are percentages.

namics assessed by ultra-deep sequencing and the clinical course of the infection has been described in patients with chronic hepatitis C treated with NS3-4 protease inhibitors [22, 23]. In our study, the analysis focused on nine well-known amino acid positions in the HBV polymerase gene that are associated with NA-resistant mutations. Because no drug-resistant mutations were detected with capillary sequencing, it is reasonable to assume that there were no mutations before treatment commencement. However, with deep sequencing, we detected several major drug-resistant mutations, including rtI169T and rtM250V conferring resistance to ETV, rtV173L to LAM, rtL180M to LAM/ETV, rtA181T to LAM/ADV/LdT, rtA181V to ADV/LdT, rtT184A/S to ETV, rtS202G to ETV, rtM204V/I to LAM/ETV/LdT, and rtN236T to ADV. The frequencies of these mutations ranged from 0.1 to 1.06% in all six samples obtained from Indonesian treatment-naïve patients with CHB (table 2). One should be careful about what threshold to use to define a clinically meaningful population. In recent studies, minority variants were defined as differences greater than 0.5 and 1.0% of mutations detected by deep sequencing [24, 25]. In this study, the proportions of the total numbers of reads containing mutations I169L/M, S202R, M204I/L or N236S were >1.0%.

Interestingly, we found no significant differences in the mutation frequencies between the chronic asymptomatic patients and the patients with advanced liver disease ($p > 0.05$; table 4). The mutation profiles revealed that these major NA-resistant mutations were present before treatment commenced and that the mutation profiles of all 6 patients were similar. In comparison with earlier studies using deep sequencing, the mutational profiles were somehow different. A study from Korea reported that minor populations present in >1% of viral quasispecies were detected in I169L, L180M and S202G in treatment-naïve patients [11], and a study from the USA revealed other mutational profiles including V173L, T184S and S202G [26]. Mutational profile differences depend on the genotype differences. In general, it was thought that clinical outcomes and prognoses differed among HBV genotypes and subgenotypes. HBV genotype B is associated with earlier HBeAg seroconversion and better response to interferon therapy than genotype C [27, 28]. Whereas HBV in our study was genotype B, other studies from Korea and the USA were genotypes C and G, respectively [11, 26].

It had been unclear whether genomic variations contributed to the emergence of preexisting NA-resistant mutations. A recent report using deep sequencing revealed that these preexisting mutations were related to therapeutic discontinuation [24]. The next-generation sequencer was first introduced in 2005 and represents a significant advance in DNA sequencing. This method reads several million to billion short DNA fragments per run [29] and more than 1 Gb of the genome can be read in one run [30, 31]. Although next-generation sequencing will inevitably

Table 3. Previously unreported mutations at nine positions associated with NA resistance detected with next-generation sequencing

Position/ substitution	Patient ID						Mean ± SD
	B13	B55	B77	B126	B143	B149	
rt169							
I169M	2.12	2.05	1.98	0.34	2.07	2.07	1.77±0.70
I169L	1.15	1.00	1.04	1.05	1.07	1.01	1.05±0.05
I169V	0.52	0.48	0.50	0.48	0.47	0.53	0.50±0.02
I169R	0.21	0.2	0.21	0.23	0.23	0.22	0.22±0.01
I169K	0.12	0.13	0.12	0.12	0.11	0.11	0.12±0.01
rt173							
V173A	0.95	0.98	0.98	0.91	0.96	0.93	0.95±0.02
V173E	0.38	0.35	0.38	0.39	0.42	0.38	0.38±0.02
V173G	0.35	0.35	0.36	0.33	0.35	0.36	0.35±0.01
V173M	0.25	0.26	0.26	0.24	0.24	0.25	0.25±0.01
rt180							
L180F	0.68	0.68	0.67	0.63	0.7	0.63	0.67±0.02
L180S	0.43	0.42	0.37	0.38	0.38	0.38	0.36±0.02
L180W	0.33	0.32	0.34	0.31	0.32	0.35	0.33±0.01
L180V	0.3	0.3	0.28	0.3	0.3	0.3	0.3±0.01
rt181							
A181S	0.32	0.32	0.33	0.31	0.36	0.32	0.33±0.02
A181D	0.18	0.19	0.18	0.21	0.18	0.18	0.19±0.01
A181P	0.16	0.18	0.16	0.17	0.16	0.17	0.17±0.01
A181G	0.14	0.16	0.12	0.16	0.11	0.14	0.14±0.02
rt184							
T184N	0.19	0.18	0.17	0.19	0.2	0.18	0.19±0.01
rt202							
S202R	1.63	1.72	1.75	1.8	1.77	1.72	1.73±0.06
S202I	0.23	0.23	0.25	0.23	0.26	0.23	0.24±0.01
S202N	0.18	0.15	0.18	0.18	0.13	0.18	0.16±0.02
S202T	0.11	0.08	0.1	0.11	0.09	0.11	0.10±0.01
rt204							
M204L	1.53	1.49	1.75	1.55	1.54	1.53	1.57±0.09
M204T	0.45	0.45	0.25	0.43	0.45	0.45	0.41±0.08
M204K	0.15	0.16	0.1	0.15	0.16	0.15	0.15±0.02
M204R	0.44	0.39	0.18	0.38	0.37	0.44	0.37±0.09
rt236							
N236S	1.04	0.95	0.95	0.95	0.97	0.98	0.97±0.03
N236D	0.45	0.45	0.44	0.45	0.42	0.43	0.44±0.01
N236I	0.05	0.43	0.45	0.49	0.42	0.49	0.39±0.17
N236H	0.33	0.36	0.35	0.34	0.34	0.35	0.35±0.01
rt250							
M250I	0.34	0.31	0.36	0.32	0.4	0.36	0.35±0.03
M250R	0.26	0.25	0.24	0.26	0.26	0.25	0.25±0.01
M250L	0.21	0.20	0.22	0.22	0.21	0.23	0.22±0.01

Values are presented as percentages.

make a significant contribution to human genomics, it is also effective in the detection of mutant populations occurring at low frequencies in specific viral genomes. In our study, the method sequenced more than 100,000 DNA fragments, allowing detailed analyses. Frequencies of 1–2% for the substitutions L180M or M204V/I in pretreatment sequences were reported for patients who subsequently developed resistance to LAM [11, 24]. Therefore, a frequency of approximately 1% for the preexisting mutations M204I (conferring resistance to LAM/LdT) and

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Table 4. NA-resistant mutations in treatment-naïve patients with HBV according to clinical conditions

Position/ substitution	NA resistance	Asymptomatic CHB, %	Advanced liver disease, %	p value
rt169/I169T	ETV	0.193	0.187	0.23
rt173/V173L	LAM	0.280	0.275	0.67
rt180/L180M	LAM/ETV	0.120	0.123	0.19
rt181				
A181T	LAM/ADV/LdT	0.330	0.342	0.28
A181V	ADV/LdT	0.360	0.363	0.72
rt184				
T184A	ETV	0.970	0.930	0.14
T184S	ETV	0.523	0.493	0.49
rt202/S202G	ETV	0.533	0.523	0.35
rt204				
M204V	LAM/ETV	0.623	0.595	0.97
M204I	LAM/LdT	0.890	1.055	0.33
rt236/N236T	ADV	0.667	0.68	0.15
rt250/M250V	ETV	0.340	0.35	0.72

T184A (to ETV) might predict the emergence of mutations conferring resistance to NAs in these patients.

In conclusion, deep sequencing is an effective method to detect minor populations of antiviral drug-resistant mutations in treatment-naïve patients with CHB. Therefore, deep sequencing is a useful method for predicting the future emergence of antiviral drug-resistant HBV during long-term NA therapy. However, the threshold of a clinically meaningful population must be evaluated in future studies. This study shows that the profiles of NA-resistant mutations in treatment-naïve patients with HBV genotype B3 differ from those in similar studies of genotype C patients [11]. Nevertheless, our findings suggest that the genomic variations in HBV might play a crucial role in the subsequent evolution of NA resistance.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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