

Putative and pretreatment drug resistance mutations in reverse transcriptase gene among untreated chronic hepatitis B patients at Arifin Achmad Regional District Hospital, Riau, Indonesia

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ABSTRACT

Background: Mutations within the hepatitis B virus (HBV) reverse transcriptase (RT) gene have been associated with drug resistance against nucleos(t)ide analogs (NAs).

Objective: This study aimed to identify mutations in the RT gene among patients with chronic hepatitis B (CHB) before receiving antiviral therapy and its relationship with the HBV genotypes.

Methods: A total of 26 HBV DNA was extracted from the blood plasma of CHB patients. HBV RT gene was amplified and sequenced using the Sanger dideoxy sequencing method. The HBV genotype was determined through phylogenetic analysis using the Maximum Likelihood method.

Results: The study subjects comprised 14 CHB patients without complications and 12 CHB patients with cirrhosis/hepatoma. CHB patients with cirrhosis/hepatoma were older than those without complications. The HBV genotypes comprised 15 (57.7%) genotype C and 11 (42.3%) genotype B. All treatment-naïve CHB patients did not demonstrate any classical NA resistance mutations within the RT gene. However, several putative and pretreatment resistance mutations, including F221Y, N238H, and V224I, were high frequency in more than 40% of study subjects. In addition, F221Y and N238H/Q mutations were frequently observed in genotype B, while C224I was only found in patients infected with genotype C ($p=0.000$).

Conclusions: There was no evidence of classical RT gene mutations associated with NA resistance in treatment-naïve patients with CHB. However, several putative and pretreatment mutations were identified as genotype-specific mutations and may contribute to antiviral resistance against NAs.

Keywords: chronic hepatitis B, genotype, nucleos(t)ide analogues, reverse transcriptase, resistance mutation.

Introduction

Globally, more than 2 billion people have been infected with hepatitis B virus (HBV) and 290

million of them have chronic hepatitis B (CHB) infection [1]. Patients with CHB are at a higher risk of developing fatal liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). About 50% of HCC is attributed to CHB infection [2]. Hepatitis B is still a major health problem in many countries, including Indonesia, despite the fact that vaccination programs against it have been in place for more than two decades. Indonesia is categorized as having a moderate endemicity for HBV infection according to the seroprevalence of hepatitis B surface antigen (HBsAg), which is 7.1% [3].

The HBV genome is a partially double-stranded DNA, consisting of four overlapping open reading

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frames (ORFs) encoding the virus polymerase, S, X, and core protein. Viral polymerase consists of four domains: a carboxy-terminal, spacer, reverse transcriptase (RT), and RNaseH domain. Reverse transcriptase is crucial for HBV replication that converts pregenomic RNA-intermediate into HBV DNA. Since the reverse transcriptase lacks the proofreading function, there is a high frequency of mutations during viral active replication, leading to genetically heterogeneous viral populations [4]. Hepatitis B mutation rates have been reported to range from 10^{-4} to 10^{-6} substitutions/site/year [5,6]. Mutations may already exist in a patient who has never received treatment (untreated) or they may develop naturally as a result of HBV infection or antiviral therapy. Mutations within the HBV RT gene are thought to influence infection persistence, liver disease progression, and resistance to antiviral therapy [7].

Current treatment for CHB patients includes interferons (IFN) and nucleos(t)ide analogs (NAs) [8]. The availability of safe and effective antiviral NA has a significant impact on the prevention of liver cirrhosis and HCC in CHB patients. NAs suppress HBV replication through reverse transcriptase inhibition, but they do not block the formation of covalently closed circular (cccDNA), a mini-chromosome in infected hepatocytes. cccDNA has a long half-life and is essential for the persistence of viral replication and the production of new virions [9]. Because NA is unable to eliminate cccDNA, antivirals must be used for a longer period to control viral replication and reduce the risk of developing advanced liver diseases such as cirrhosis and HCC. As a result, it increases risk of the emergence of mutant viruses that are resistant to NAs. These HBV mutants pose a greater challenge for treatment of patients with CHB infection [4,10].

To date, five NAs have been approved to treat patients with CHB, including lamivudine (LMV), telbivudine (LdT), adefovirdipivoxil (ADV), entecavir (ETV), and tenofovir (TDF). Classical mutations consist of primary and secondary drug resistance mutations. Primary mutations are amino acid substitutions that reduce viral susceptibility to NAs, while secondary (compensatory) mutations

are responsible for restoring viral replication caused by primary drug resistance mutation and may reduce susceptibility to NA therapy. Reverse transcriptase mutations that have not been confirmed experimentally *in vitro* are classified as putative drug resistance mutations, while mutations found in pretreatment patients for which occurrence and drug resistance has not yet been established are referred to as pretreatment mutations [11].

HBV isolates have been divided into eight genotypes (A-H) and two putative genotypes based on more than 8% genomic sequence divergence (I-J). Geographic and ethnic distributions of HBV genotypes are diverse. The two most prevalent genotypes in Indonesia are genotype B and C [12]. Geographical distribution and antiviral drug resistance are linked to the genetic variability of HBV. Therefore, we examined drug resistance mutations across HBV reverse transcriptase gene among treatment-naïve (had not yet received therapy) CHB patients in Regional District Arifin Achmad Hospital, Pekanbaru, Riau, Indonesia. We also compared the distribution of these mutations between genotypes B and C in patients with CHB.

Methods

Patients

Twenty-six CHB patients were recruited from the Regional District Arifin Achmad Hospital's outpatient clinic from March to September 2019. The inclusion criteria included patients with CHB who were more than 18 years old, positive for both HBV DNA and HBsAg, and who had not previously received NA. Patients having co-infection with the human immunodeficiency virus and chronic hepatitis B were excluded from this study. The study participants were divided into two groups: CHB patients with and without complications from hepatoma or liver cirrhosis. The clinical diagnosis of liver cirrhosis and hepatoma was based on Ultrasonography (USG) or Computed Tomography (CT) scan or Transient Elastography (TE). A commercial kit was used to measure the levels of albumin, bilirubin, and serum ALT.

This study was approved by the Ethics Committee of Faculty of Medicine, Universitas Riau (Decree no. B/086/UN19.5.1.1.8/UEPKK/2020). Each patient provided their written informed consent, which included as consent for the collection of blood samples and the analysis of mutations as well as clearance to use clinical data from the medical record for publication.

HBV DNA extraction, reverse transcriptase (RT) gene amplification and sequencing

HBV DNA was isolated from 200 μ L of blood plasma using the Viral Nucleic Extraction Kit II (Geneaid, Taiwan), according to the manufacturer's protocol. HBV reverse transcriptase gene was amplified using the nested polymerase chain reaction (PCR) method using the following outer forward primer P1 (5'-AGTCAGGAAGACAGCCTACTCC-3') and outer reverse primer P2 (5'-AGGTGAAGCGAAGTG CACAC-3'), and inner forward primers P3 (5'-TTCCTGCTGGTGGCTCCAGTTC-3') and inner reverse primer P4 (5'-CCGCAGTATGGATCGGCAG-3') [13]. The PCR reaction was carried out in a reaction mixture of GoTaq® reaction buffer (pH 8.5), 200 μ M dNTP, 1.5 mM of $MgCl_2$, and 5 mL of HBV DNA. PCR was performed with the following conditions for the first PCR: 10 cycles of denaturation at 94°C for 35 seconds, annealing at 59°C for 35 seconds, extension at 72°C for 70 seconds, and 30 cycles of denaturation at 94°C for 35 seconds, annealing at 56°C for 35 seconds, extension at 72°C for 70 seconds; while the second PCR's conditions consisted of denaturation at 94°C for 5 minutes, then 35 cycles of denaturation at 94°C for 25 seconds, annealing at 56°C for 25 seconds and extension at 72°C for 50 seconds. The PCR products were subjected to electrophoresis on 1% agarose gel containing GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) submerged in 1x Tris Acetate EDTA (TAE) buffer at 75 volts for 40 minutes. PCR fragments were visualized in a UV GelDoc® (Bio-Rad, USA). The RT gene was sequenced using Sanger's dideoxy DNA sequencing method at 1st BASE-Apical Scientific (Selangor, Malaysia).

Reverse transcriptase mutation analysis and HBV genotyping

The RT gene sequences were trimmed, and consensus sequences were retrieved using the Snapgene program (<https://www.snapgene.com/>). The amino acid sequences were obtained using an online translation tool (<https://web.expasy.org/translate/>); the results were then aligned with HBV protein sequences deposited in GenBank using MegaX software ver. 01 (<https://www.megasoftware.net/>). The HBV genotypes were determined using an online genotyping tool (<https://hbvdb.lyon.inserm.fr/HBVdb/HBVdbIndex>) and confirmed by phylogenetic analysis using the Maximum Likelihood method, which was performed using MegaX software ver. 01.

Statistical analysis

Categorical data were expressed as a proportion, while the numerical variable was presented as mean \pm SD (min-max). *Chi-squared* test was used to examine group differences for RT mutation prevalence. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS statistic version 22 (IBM Corp., Armonk, NY, USA) for Mac.

Results

Patient characteristic

Study subjects consisted of 14 CHB patients without complication and 12 CHB patients with cirrhosis/hepatoma (Table 1). In comparison to CHB patients without complications, the mean age of CHB patients with cirrhosis/hepatoma was higher. Compared to CHB patients without complications, patients with cirrhosis/hepatocellular carcinoma had significantly higher levels of alanine aminotransferase (ALT). Similar to this, subjects with cirrhosis/hepatoma had levels of total bilirubin that were three times higher than those without complications. Conversely, CHB patients with cirrhosis/hepatocellular carcinoma had lower albumin levels than CHB patients with no complications. In addition, regardless of the

Table 1. Characteristic of study subjects

Variable	CHB without complication (n=14)	CHB with cirrhosis/ hepatoma (n=12)
Age (mean \pm SD, min-max), years	37.15 \pm 15 (21-67)	51.45 \pm 10 (39-65)
Sex		
• Male, n (%)	5 (35.7)	9 (75)
• Female, n (%)	9 (64.3)	3 (25)
ALT (mean \pm SD, min-max), IU/L	29.8 \pm 16.9 (10-63)	84.6 \pm 64.3 (15-203)
Total bilirubin (mean \pm SD, min-max), mg/dL	0.7 \pm 0.6 (0.3-2.5)	2.8 \pm 3 (0.5-10.9)
Albumin (mean \pm SD, min-max), g/dL	4.1 \pm 0.7 (2.5-4.7)	3.1 \pm 0.7 (2-4.2)
HBV genotype		
• Genotype B	6 (42.9)	5 (41.7)
• Genotype C	8 (57.1)	7 (58.3)

CHB: Chronic hepatitis B

Table 2. Prevalence of non-classical NA resistance mutation among chronic hepatitis B patients

Mutation category	Mutation	CHB without complication (n=14) (%)	CHB with cirrhosis/ hepatoma (n=12) (%)	P value
Putative drug resistance mutation	S213T	0 (0)	1 (8.3)	0.462
	Q215H	0 (0)	1 (8.3)	0.462
	E218D	2 (14.3)	1 (8.3)	0.636
	F221Y	7 (50)	5 (41.7)	0.976
	L229V	1 (7.1)	0 (0)	0.462
	N238H/Q	7 (50)	5 (41.7)	0.624
Pretreatment mutation	V224I	7 (50)	7 (58.3)	0.976

CHB: Chronic hepatitis B

Table 3. The distribution of non-classical NA resistance mutation based on HBV genotypes

Mutation category	Mutation	Genotype B (n=11) (%)	Genotype C (n=15) (%)	P value
Putative drug resistance mutation	S213T	1 (9.1)	0 (0)	0.423
	Q215H	0 (0)	1 (8.3)	
	E218D	0 (0)	3 (20)	0.238
	F221Y	11 (100)	1 (8.3)	0.000
	N238H/Q	11 (100)	1 (8.3)	0.000
Pretreatment mutation	V224I	0 (0)	14 (93.3)	0.000

presence of complications, genotype C of HBV predominated over genotype B in both groups (Table 1). Figure 1 depicts the evolutionary tree of the hepatitis B RT gene.

Distribution of drug resistance mutations among CHB patients

We examined the amino acid changes linked to NA resistance in RT protein sequences spanning

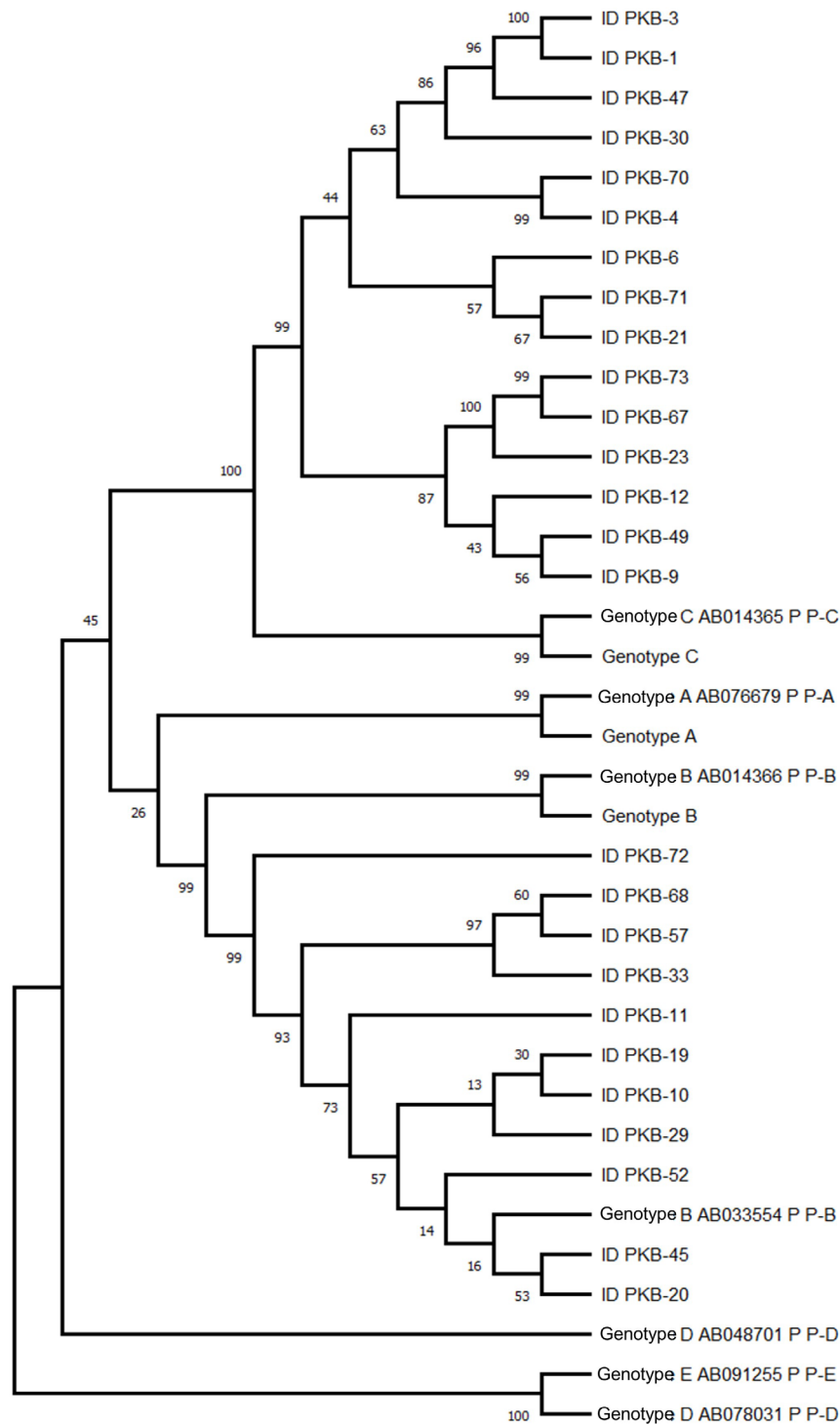


Figure 1. Phylogenetic tree analysis of reverse transcriptase gene of hepatitis B

amino acids E1 to P356 and divided them into primary, secondary (compensatory), putative, and pretreatment mutations. We did not find any primary mutations (I169M, A181T/V/S/G, T184K/L/I/T/P/S/A, S202G, M204I/V, N236T/V/K/I/A, and M250L/V) or secondary mutations (V173L/M and L180M/Q) related with NA resistance. In contrast,

several putative and pretreatment mutations were detected in patients with CHB who had not had treatment before (treatment-naïve patients) (Table 2). Among putative mutations, F221Y and N238H/Q mutations were observed in more than 40% of subjects in both groups. E218D mutation was found in both CHB patients without and with

complications, with a prevalence of 14.3% and 8.3%, respectively. L229V was found in one of the CHB patient without complications, whereas S213T and Q215H were identified in one CHB patient with cirrhosis/hepatoma. Additionally, we found that more than 50% of CHB patients had the pretreatment mutation V224I. Between CHB patients with and without cirrhosis/hepatoma complications, there was no significant difference in the frequency of any of the aforementioned mutations.

Table 3 shows RT amino acid mutations among pretreatment CHB patients based on HBV genotypes. Of seven putative and pretreatment mutations detected, F221Y and N238H/Q were found in all subjects with genotype B, but these mutations only found in one in patient with genotype C ($p < 0.001$). In contrast, pretreatment mutation, V224I, was only found in CHB patients with genotype C (93.3%), and there was a significant difference in the RT mutation frequency between genotype B and C ($p < 0.001$).

Discussion

Although effective antiviral therapy is available for treating chronic hepatitis B, lengthy treatments are frequently necessary since NAs are unable to eliminate cccDNA from infected hepatocytes. The lack of proofreading function of HBV polymerase underlies the increased accumulation of deleterious mutations, including drug resistance mutations, during HBV replication [14]. This study analyzed preexisting drug resistance mutations across RT protein sequences in 26 CHB patients. The results showed no common NA resistance mutations within the RT gene among treatment-naïve patients with CHB. It indicates there is no naturally occurring mutations in our study population, and drug resistance mutations are more likely due to drug pressure selection during antiviral treatment [15].

There were limited published data reporting the prevalence of drug resistance mutations among Indonesian CHB patients in particular prior to receiving antiviral treatment, and our study would

add to the data regarding drug resistance mutations in Indonesia. While no classical drug resistance mutations were found in our study, Yamani et al. [7] reported the prevalence of primary (A194T and M204V/I) and secondary mutations (L80V and L180W/M) in 8.3% of CHB patients before initiating antiviral treatment. They analyzed 96 Indonesian RT gene sequences deposited in GenBank. M204V/I mutation is a frequent amino acid change in the YMDD motif and is responsible for reduced therapeutic response against lamivudine by more than a 100-fold decrease compared to wild-type. Lamivudine, the first NA agent used in clinical practice, has demonstrated effective suppression of HBV DNA but has a low barrier against resistance mutations. Consequently, a high rate of drug resistance mutations—up to 50% in a 5-year study—has been observed in individuals taking lamivudine monotherapy [16].

A recent study by Fu et al. reported that only 1.1% of CHB patients harbored classical drug resistance mutations. Preexisting drug resistance mutation incidence in CHB patients ranged from zero to more than 5% [7,15]. This discrepancy may be caused by a number of factors, such as geographic location, ethnic background, or viral genotype. An epidemiological study in multi-ethnic regions from Western China reported that adefovir resistance in association with A181T/V was more frequently found in genotype C patients than genotype B patients [17]. The distinct geographical distribution of antiviral resistance has also been identified in a study demonstrating North America as one of the regions with the highest frequency of drug resistance mutations against NAs [18]. Additionally, Sanger Sequencing's inability to identify rare mutations may cause population-wide minor changes to go undiscovered. Therefore, ultrasensitive and high throughput sequencing methods, such as deep sequencing [19] and PCR-based CRISPR-Cas13a systems [20], are required to detect these minor strains.

A number of putative and pretreatment mutations were identified in the RT sequences by this study, and interestingly, three of these mutations—F221Y, N238H, and V224I—were found to occur frequently

in more than 40% of subjects. S213T, Q215H, E218D, and L229V were among the other putative and pretreatment mutations, however they were found far less frequently. According to a previous study, these mutations were found in patients who developed viral breakthroughs during receiving NA treatment [21]. The evidence from *in vitro* study is currently extremely scant, thus more study is still needed to determine the implications of these mutations on viral replication. The development of drug resistance in treatment-naïve patients with only putative RT mutations and no primary or secondary mutations has also been reported in a number of studies [22].

Similar to the previous findings [23], genotype B (42.3%) and genotype C (57.7%) made up the majority of the HBV genotype distribution in this study. The genotype distribution in this study was different from the dominant genotypes reported in several other regions in Indonesia, where genotype B is generally found. In a previous study involving 28 Indonesian cities, genotype B was shown to be the predominant genotype (66%) and to be followed by genotypes C (26%), D (7%), and A (0.8%) [24].

In the present study, we identified two putative and one pretreatment genotype-specific mutation within RT sequences. All subjects with genotype B displayed F221Y and N238H/Q mutations, in contrast to only one of those with genotype C. These findings differed from the previous research by Yamani et al. [7] and Zhang et al. [21], which found that genotype C was more likely to carry the F221Y mutation than genotype B ($p=0.000$). The AB domain of the RT of genotype C tended to have mutations compared to genotype B, according to a large-scale analysis of ~6500 full-length HBV [18]. Likewise, a Chinese study found that patients with HBV genotype C were more likely to have preexisting drug-resistant mutations than patients with HBV genotype B [25].

The clinical significance value of non-classical mutations in clinical practice remains unknown, which may be due to relatively low frequency and lack of phenotypic evidence from *in vitro* studies

[21]. It should be noted that a number of putative or pretreatment mutations found in treatment-naïve patients, including F221Y and V224I, have been linked to advanced liver diseases such liver cirrhosis and HCC [11]. Furthermore, F221Y mutation was demonstrated to be an independent risk factor for the HCC prognosis following liver resection [15]. It is possible for mutations in the RT and HBsAg regions to occur simultaneously since the RT gene and the hepatitis B surface antigen (HBsAg) gene sequences partially overlap. Preexisting RT mutations typically have a non-random distribution and are most frequently observed in the A-B interdomain, which overlaps with the HBsAg “a” determinant region. These mutations may lead to the emergence of immune escape “a” variants, which may contribute to the persistence of infection [11].

Overall, these findings indicate that the distribution of drug resistance mutations within the RT domain in treatment-naïve patients may potentially affect viral fitness and may therefore play a role in the emergence of HBV mutants that are resistant to antiviral treatment. Due to the small number of study participants included in this study, further research is necessary to determine the impact of RT mutations in the emergence of drug resistance against NAs.

Conclusion

No evidence of primary and secondary drug resistance mutations within RT gene in treatment-naïve patients with CHB. However, F221Y and N238H/Q of RT gene were identified as genotype-specific mutations and may contribute to antiviral resistance against NAs. This characterization of drug resistance mutations in treatment-naïve is useful to tailor antiviral treatment strategy for patients with CHB during prolonged use of antiviral therapy.

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Author contributions

AA, FAD, and MM conducted data collection and wrote the manuscript. HA, DKS and TORW contributed to the completion of the manuscript (intellectual input). AA obtained funding, provided guidance to study design, supervised study, reviewed and finalised the manuscript.

Declaration of interest

The authors have no conflict of interest to disclose.

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