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Retrospective Analysis of HBV Pre-Existing Mutations and Drug-induced Resistance Mutations in Patients with Hepatitis B Virus-Related Cirrhosis

Wang Liping*, Dai Mingjia, Li Chunyang, Hao Jungui, Ji Fang and Yan Xuebing

Department of Infectious Diseases, Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, China

***Correspondence:** Yan Xuebing, Department of Infectious Diseases, Affiliated Hospital of Xuzhou Medical University, 9 North Kunpeng Road, Xuzhou, Jiangsu, China 221000, E-mail: yxbxuzhou@126.com

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Abstract

Background: The reported prevalence and necessity of detection of HBV reverse transcriptase (RT) mutation prior to treatment is varied and remains controversial. This study aimed to identify the prevalence of HBV pre-existing gene resistance mutations and compare the difference between pre-existing mutations and drug-induced resistance mutations in patients with hepatitis B virus-related cirrhosis.

Methods: 180 patients with hepatitis B virus-related cirrhosis which included 68 patients with virological breakthrough and 112 treatment-naive cirrhosis patients were retrospectively enrolled. The drug-resistant mutations of HBV reverse transcriptase domain were screened by direct gene sequencing. One-way ANOVA analysis was performed in the comparison among different groups. Ratios difference was compared with the chi-square test.

Results: There were 48 patients (48/112, 42.86%) with drug resistance mutations in nucleoside/nucleotide analogues (NAs) treatment-naive group, 59 patients (59/68, 86.76%) showed drug-resistant mutations in the NAs treatment group. The gene resistance mutation patterns in treatment-naive group were mainly rtS213T, rtV214A, 191V/I and rtN/H238T/D, and the types of resistance mutations in the treated group were different. The adefovir (ADV) group: mainly rtA181T/V and rtS213T; lamivudine/ telbivudine (LAM/LDT) group: rtL180M+ rtM204I/V/S and rtM204I/V/S or a complex mutation pattern containing 204 site; entecavir (ETV) group: The drug resistance pattern is the simultaneous presence of multiple site mutations. LAM/LDT sequential ADV group: The variant type was multi-site and resistant to both ADV and LAM.

Conclusion: There was a prevalence of pre-existing mutations in RT region of HBV polymerase in patients with hepatitis B virus-related cirrhosis, The mutation pattern is mainly related to LAM and ADV-related compensatory mutations, while the drug-induced mutation pattern is more complicated, mainly related to the antiviral drugs used and there are mainly primary mutations. Patients with cirrhosis should be tested genetic resistance mutation before using antiviral drugs.

Keywords: Hepatitis B virus, Cirrhosis, Pre-existing drug resistance, Mutation, Reverse transcriptase, Nucleos(t)ide analogues (NAs)

Abbreviations: LAM: Lamivudine; ADV: Adefovir; LDT: Telbivudine; ETV: Entecavir; TNF: Tenofovir; HBV: Hepatitis B virus; RT: Reverse Transcriptase; NAs: Nucleoside and Nucleotide Analogs; CHB: Chronic Hepatitis B; HCC: Hepatocellular Carcinoma; LC: Liver Cirrhosis; ALT: Alanine Aminotransferase; TB: Total Bilirubin; ALB: Albumin

Introduction

Hepatitis B virus (HBV) infection leads to chronic hepatitis B (CHB), cirrhosis and even hepatic carcinoma. Antivirus therapy is the only way to inhibit virus reproduction and delay the disease progression. Currently the mature antivirus methods include limited-course interferon treatment and unlimited-course nucleoside/nucleotide analogues (NAs) treatment. NAs include lamivudine (LAM), adefovir dipivoxil (ADV), Telbivudine (LDT), entecavir (ETV) and tenofovir disoproxil fumarate (TNF) etc. However, virus resistance mutation is also an unavoidable problem in the application of NAs drugs. Once the virus resistance mutation occurs, it means that the efficacy of the antiviral drug is reduced or invalid, and the patient's condition may further deteriorate. Previous studies on genetic variation and drug resistance in HBV RT region were showed in table 1 [1-8]. Due to the highly efficient replication of the HBV DNA polymerase encoded by the HBV-encoding reverse transcriptase region, HBV reverse transcriptase lacks proofreading activity, and due to its lack of 3'-5' exonuclease activity, mRNA is intermediate during replication. When reverse transcription is replicated, base pairing errors are prone to occur. This mismatch rate is much higher than other DNA viruses, which leads to the high mutation of HBV gene, which leads to drug resistance, which may be the cause of pre-existing drug resistance. The question is whether the viral resistance mutations we encounter in our clinic are pre-existing or drug-induced? Since there is the possibility of pre-existing drug resistance, is it necessary to detect HBV gene resistance mutation before application of nucleoside drugs? Is there any difference between pre-existing mutations and drug-induced drug resistance mutations especially in liver cirrhosis patients? Based on these ideas we have established this retrospective research.

Materials and Methods

Patients

This study was retrospective. The serum samples of 180 patients with hepatitis B virus-related cirrhosis in this study were collected from the outpatients and hospitalized patients of the Affiliated Hospital of Xuzhou Medical University from June 2011 to December 2015, including 141 males and 39 females, aged 20-80 years old and the average age was 48.82±11.89 years. 112 hepatitis B virus-related cirrhosis patients without NAs treatment, 68 patients with cirrhosis who had virological breakthrough with nucleoside antiviral therapy or who had poor response for more than half a year, The diagnostic criteria were based on 2015 Guidelines for the Prevention and Treatment of Chronic Hepatitis B [9], exclusion criteria were as follows: overlapping infection of hepatitis A, C, D or E; EB virus and HIV infection; cytomegalovirus infection; combining with alcoholic liver disease or autoimmune diseases.

Instruments and reagents

7500 real-time PCR system (Applied Biosystems, Darmstadt, Germany); 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA); HBV reverse transcriptase (RT) region (covered B domain to E domain) is amplified using a forward primer: 5c-GTATGTTGCCCGTTTGTCCTC-3c (nt459~479); and a reverse primer 5c-CCCCAACTTCCAATTACATAT-3c (nt882~902), it covered common mutations from HBV RT region B domain to E domain. Primers were synthesized by Shenyou Biological Engineering Company, Shanghai, China. PCR amplification and sequencing reagents were bought from Shenyou Biological Engineering Company, Shanghai, China.

Methods

HBV DNA template was prepared by following the protocol of extraction kit (Hepatitis B Virus and Drug Resistance Related Mutation Detection Kit. REG. NO: SFDA (I) 20093400185). HBV genotyping and resistance locus mutations were performed using the Web-based National Center for Biotechnology Information (NCBI) retrovirus genotyping analysis platform (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) (we just performed according to the instruction on the website)

Statistical analysis

SPSS16.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. One-way ANOVA analysis was performed in the comparison among different groups. Ratios difference was compared with the chi-square test. A *P* value of less than or equal to 0.05 was considered statistically significant.

Results

HBV genotype and clinical features

Samples from 180 liver cirrhosis patients were enrolled in this retrospective study. Through gene sequencing and sequence alignment with 10 standard genotype (A-J) strains in Genbank, HBV genotypes of the subjects were determined with 7 cases (3.9%) type B and 173 cases (96.1%) type C. No other genotypes were detected. There

were no significant differences in age, gender, and ALB levels between the two genotypes ($P > 0.05$). The ALT and TB were significantly higher in the gene B type than in C genotype group, and baseline serum HBV DNA in gene type B group was also significantly higher than that in C genotype group. There were statistical differences between the two groups (Table 2).

Table 1: HBV RT gene mutation and associated drug resistance.

Drug resistance	Mutations in RT region
LAM	rtV173L, rtL180M, rtA181V/T/S, rtS-202G/I , rtM204I/V/S, rtV207I/L/G, rt-S213T, rtQ215E/H/P/S, rt191V/I, rtA200V, rtL 229G/V/W/F
ADV	rtA181V/T/S, rtA194T, rtM204I/V/S, rtV214A, rtQ215E/H/P/S, rtN236T, rtP237H, rtN/H238T/D, rt191V/I, rt-221F/Y
ETV	rtL180M, rtT184A/G/I/S, rtS202G/I, rt-M204I/V/S, rtS213T , rtM250V/L
LDT	rtL180M, rtM204I/V/S, rt191V/I
TNF	rtP177G, rtA194T, rtM204I/V/S, rtF249A, rtA181V/T/S, rtN236T
Unknown	rt178F/Y, rt224I/V

ADV: Adefovir dipivoxil; ETV: Entecavir; LDT: Telbivudine; LAM: Lamivudine; TNF: Tenofovir

Table 2: HBV genotype and clinical features of enrolled patients.

Items	Genotype B(n=7)	Genotype C (n=173)	P
Age in yrs (mean ± SD)	40.43±17.73	49.16±11.54	0.076
Gender (male/female)	7/0	134/39	0.062
ALT(U/L)	392.43±346.53	135.44±174.49	0.001
TB(umol/L)	134.86±211.36	71.29±109.98	0.002
ALB(g/L)	39.60±6.17	33.66±8.05	0.289
HBV DNA (log10IU/ml)	5.06±2.12	5.77±1.33	0.027
mutation(%)	1(3.9%)	68(96.1%)	0.002

The positive distribution of HBeAg in the antiviral treatment group and the treatment-naive group

Regarding the distribution of e antigens, since the number of B genotypes case was small, no statistical analysis was performed. There was no significant difference in the positive distribution of e antigen between the treated group and the treatment-naive group in HBV C genotype ($P = 0.05$) (Table 3).

Table 3: HBeAg positive distribution in antiviral group and treatment-naive group.

HBV genotype	B		C		total	χ2	P
HBeAg	+	-	+	-			
treated	1	5	54	52	112		
treatment-naive	0	1	32	35	68	5.55	0.05

The relationship between HBV genotype and viral gene resistance mutation

In 180 patients with cirrhosis, 102 cases of known site-specific gene resistance mutations occurred, and 5 cases of unknown drug-resistant site mutations were found, except one case of mutation occurred in B genotype, and the rest occurred in C genotype. The results were list in table 4 and 5.

Table 4: HBV gene mutation pattern in treatment-naive group.

genotype	mutation type	case	Resistant drug
C	rtV173L	2	L
C	rtA181T/V	1	L/A/T
C	rtM204I/V/S	1	L/A/E/T
C	rtV207I/L/G	1	L
C	rtS213T	7	L/E
C	rtV214A	7	A
C	rtQ215S	2	L/A
C	rtN/H238T/D	4	A
C	rtL180M+rtS213T	1	L/E
C	rtA181T/V+rtV214A	1	L/A/T
C	rtV207I/L/G+ rtS213T	1	L/E
C	rtS213T+ rtN/H238T/D	1	L/A/E
C	191V/I	7	L/A
C	229L/V	1	L
C	224I/V	3	UN
C	221F/Y	3	A
C	191V/I+221F/Y	4	L/A
C	191V/I+221F/Y+229L/V	1	L/A
Total		48	

Resistant drug: L: Lamivudine; A: Adefovir dipivoxil; E: Entecavir; T: Tenofovir; UK: Unknown

Characterization of pre-existing mutations in RT

Table 5: HBV gene mutation pattern in NAs treated group.

genotype	mutation type	Total case	Treated group				Resistant drug
			A	B	C	D	
C	rtA181T/V	3	2	0	0	1	L/A/T
C	rtT184I	1	1	0	0	0	L/E
C	rtM204I/V/S	9	0	4	0	5	L/A/E/T
C	rtV207I/L/G	1	1	0	0	0	L
C	rtS213T	3	3	0	0	0	L/E
C	rtV214A	1	0	0	0	1	A
C	rtN/H238T/D	1	0	0	0	1	A
C	rtV173L+ rtL180M+ rtM204I/V/S	2	1	0	0	1	L/A/E/T
C	rtL180M+ rtM204I/V/S	6	0	6	0	0	L/A/E/T
C	rtL180M+ rtA181T/V+ rtM204I/V/S	1	0	0	0	1	L/A/E/T
C	rtL180M+rtA181T/V+rtM204I/V/S+ rtV207I/L/G	1	0	1	0	0	L/A/E/T
C	rtL180M+rtT184A/G/I/S+rt-M204I/V/S	1	0	0	1	0	L/A/E/T
C	rtA181T/V+ rtM204I/V/S	1	0	1	0	0	L/A/E/T
C	rtA181T/V+ rtM204I/V/S+rtV214A	1	0	1	0	0	L/A/E/T
C	rtA181T/V+ rtM204I/V/S+ rtN236T	2	0	0	0	2	L/A/E/T
C	rtA181T/V+rtV207I/L/G+rtN236T+ rtN/H238T/D	1	0	0	0	1	L/A/T
C	rtA181T/V+rtV214A	1	0	0	0	1	L/A/T
C	rtA181T/V+ rtN236T	1	1	0	0	0	L/A/T
C	rtA181T/V+ rtN/H238T/D	2	2	0	0	0	L/A/T
B	rtM204I + rtV207L	1	0	1	0	0	L/A/E/T
C	rtM204I + rtV214A	1	0	1	0	0	L/A/E/T
C	223S/A	2	1	1	0	0	A
C	229L/V	3	1	0	0	2	L
C	178F/Y	1	0	1	0	0	N
C	191V/I	6	5	0	0	1	L/A
C	191V/I+ 224V/I	1	0	0	0	1	L/A
C	221F/Y+ 229L/V	1	0	0	1	0	L/A
C	191V/I +221F/Y+229L/V	1	1	0	0	0	L/A
C	191V/I + 229L/V	1	0	1	0	0	L/A
C	223S/A+191V/I+229L/V	1	0	1	0	0	L/A
C	224I/V	1	1	0	0	0	UK
total		59	20	19	2	18	

regions from treatment-naive cirrhosis patients

From 112 treatment-naive cirrhosis patients, 14 samples (12.5%, 14/112) of ADV single-drug resistance mutations were detected, which were rtV214A in 7 cases (3.6%, 7/112), 4 rtN/H238T/D (3.1%, 4/112), 3 rt221F/Y (2.7%, 3/112). There were 4 cases (3.6%, 4/112) of LAM-related single drug resistance mutations, 2 rtV173L (1.8%, 2/112), 1 rtV207I/L/G (0.9%, 1/112), 1 rt229L/V (0.9%, 1/112), 9 samples (8.0%, 9/112) resistance to LAM and ETV, and 14 samples with different degrees of resistance to LAM and ADV (12.5%, 14/112) (Table 4).

Characterization of mutations in RT regions

from NAs treated cirrhosis patients

In the ADV treated group, 20 cases of gene resistance mutations were detected (29.4%, 20/68), mainly 6 cases (8.8%, 6/68) resistant to LAM/ADV, and 5 cases of LAM/ADV/TNF resistance (7.4) %, 5/68). There were 19 cases of gene resistance mutation in LAM treated group (27.9%, 19/68), 14 cases of LAM/ADV/ETV/TNF multidrug resistance (20.6%, 14/68). 2 cases of gene mutation were detected in ETV treated group (2.9%, 2/68); 18 cases (26.5%, 18/68) of drug-resistant mutations were detected in the LAM/ADV (LAM and ADV sequential treated) group, mainly 9 cases (13.2%, 9/68) of LAM/ADV/ETV/TNF multidrug resistance. In addition, rt224I/V locus variation was detected in both the treated and untreated groups, and 1 178F/Y variant was also detected in the LAM-treated group (Table 5).

Comparison of genetic resistance mutation rates between antiviral treatment group and treatment-naive group

In 112 cases of treatment-naive group, 48 cases (42.86%) of mutations were detected, in 68 cases of antiviral treatment group there were 59 cases mutations detected (86.76%). The mutation rates of the two groups were statistically different. $P = 0.00$ (Table 6).

Table 6: The mutation rate between the treated and untreated patients.

Mutation	untreated	treated	χ2	P
yes	48	59		
no	64	9		
Mutation rate	42.86%	86.76%	33.84	0

Discussion

Hepatitis B virus (HBV) resistance mutation is a major obstacle to the antiviral efficacy of nuleoside (acid) drugs. Studies have shown that HBV gene resistance mutations are associated with infected HBV genotypes [8, 10]. Some scholars believe that HBV C genotype is more prevalent resistant than B genotype [11, 12], but some scholars have found that A181T/V mutation, ETV resistance mutation and multidrug resistance mutation is more predominant in C genotype than B genotype, The incidence of M204I and N236T mutation in B genotypes was higher than that of in C genotype [13]. Some scholars believe that the treatment responses of NAs are similar in each genotype, but there are differences among the primary resistance mutations [14]. In this study, Among 180 cases of LC patients, 102 cases were detected known gene resistance mutations, 5 cases of unknown mutations, except one case of mutation occurred in HBV B genotype, the rest of mutations occurred in the C genotype. Therefore, the results of this study suggest that HBV gene resistance mutations are associated with HBV genotype, and HBV genotypes C are more susceptible to mutation than genotype B. The levels of HBV DNA loads in patients of genotype B was lower than that of C genotype, while ALT and TB levels are higher than those of C genotype, suggesting that the level of viral replication in patients with cirrhosis infected with HBV B genotype may be low, but liver damage may be more serious than that of C genotype.

HBV pre-existing gene resistance has been confirmed by many studies [15-17], and the pre-existing mutation patterns of gene resistance are diverse [15]. However, there is no consensus on HBV genetic resistance testing before NAs antiviral treatment. Some scholars believe that the prevalence of pre-existing drug resistance is low, and there is no need for routine drug resistance mutation detection before starting antiviral therapy [18, 19]. Our previous study found that there is a pre-existing gene resistance mutation in chronic hepatitis B. The occurrence of genetic drug resistance mutations is related to the infected HBV genotype. As the disease progresses, there seems to be an increasing trend in preexisting drug resistance mutations rates [20].

In the 112 patients with LC who were not treated with antiviral drugs, 48 (48/112, 42.86%) of the gene resistance mutations were detected, and 59 cases in 68 of the antiviral treatment groups detected genetic resistance mutations (59/68, 86.76%), there was a statistically significant difference in the incidence of mutations between the two groups. Although the presence of pre-existing gene resistance mutations in the natural state is lower than drug-induced gene resistance. However, the incidence of genetic drug resistance mutations in patients with cirrhosis is as high as 42.86%, so patients with HBV-related cirrhosis should be tested for genetic resistance before using NAs antiviral therapy.

The difference in gene mutation sites has different effects on the ability of HBV to replicate and its antiviral effect on NAs. The amino acid changes in the RT region caused by the nucleotide variation of the HBV polymerase gene, such as rtA181T/V, rtM204V/I/S, rtN236T and rtM250I/V, have decreased HBV replication ability and sensitivity to NAs. the variation of these loci is called the main resistance mutation or the primary resistance

mutation, and rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184A/C/G/S, rtS202C/G/I, rtV214A, rtQ215S, Variants of rtL217P, rtL229M, rtI233V and rtN238H are often mutated on the basis of primary resistance mutations that partially restore the replication ability of the variant virus or further lead to the sensitivity of the variant virus to the drug, The mutations are considered to be a secondary variation or a compensation variation [18, 21, 22]. Rt214, rt215, rt221 and rt238 variants can reduce the efficacy of ADV antiviral and are therefore referred to as "secondary variants" or "proposed variants" [23-25].

Our results showed that the drug resistance mutations of the untreated group were mainly rtS213T, rtV214A, rt191V/I and rtN/H238T/D, and the resistance mutations after treatment were mainly primary resistance mutations. ADV group: mainly rtA181T/V single site point and multi-point variation containing this site and rtS213T and rt191V/I mutation; LAM/LDT group: rtL180M+ rtM204I/V/S and rtM204I/V/S or containing 204 sites multi-site variation patterns; ETV group: rtL180M + rtM204I/V/S+ rtT184A/G/I/S and 221F/Y+ 229L/V variant. LAM/LDT sequential ADV group: The variant type is mainly rtM204I/V/S or multi-site variation with 204 sites, and contains 229L/V single-site and multi-site variation.

From the results of the study, the genetic resistance mutation patterns of the treated group and the untreated group of LC patients are different. Does the genetic mutation pattern affect the existing NAs efficacy?

Previous studies have shown that the rtS213T variant has some degrees of resistance to LAM and ETV [26, 27]. This study found that 8.9% (10/112) of patients with hepatitis B-related cirrhosis without antiviral therapy contained Single point variation and multiple point variation of this site. This also means the presence of pre-existing resistance to ETV in patients with cirrhosis. The rtV214A mutation has been shown to be resistant to ADV [28], and the rtF221Y mutation associated with ADV resistance is thought to be an independent risk factor for liver cancer [29]. The rtL229 mutation may be associated with LAM resistance, which is a compensatory variant of the rtM204I mutation [30]. Our results suggest that rtL229 occurs primarily in patients treated with LAM or LAM for ADV, This site mutation also occurs in NAs treatment-naive cirrhosis patients.

In the guidelines for the treatment of chronic hepatitis B, it is recommended to use entecavir 1 mg instead of 0.5 mg in patients with hepatitis B associated decompensated cirrhosis [31, 32]. Is it the poor efficacy of ETV in patients with hepatitis B associated cirrhosis due to its high prevalence preexisting resistance? If this is the case, it is necessary to conduct a genetic resistant mutations test before the selection of antiviral drugs in patients with hepatitis B associated decompensated cirrhosis. Of course, the number of cases we have studied is relatively small. And further research is needed to check its authenticity.

Conclusion

In conclusion, there was a prevalence of pre-existing mutations in RT region of HBV polymerase in patients with hepatitis B virus-related cirrhosis, although the prevalence of drug resistance was lower than that induced by NAs (42.86% vs 86.76%), the overall incidence was relatively high, and the pattern of pre-existing gene resistance mutations was mainly related to LAM and ADV-related compensatory mutations, and there are also minor mutations associated with ETV resistance. while the drug-induced mutation pattern is more complicated, mainly related to the antiviral drugs used and there are mainly primary mutations. Patients with cirrhosis should be tested genetic resistance mutation before using antiviral drugs.

Declarations

Ethics approval and consent to participate

Retrospective review of anonymized data from the patients was approved by Ethics Committee of Xuzhou Medical University Affiliated Hospital (xyfylw2013026) and all aspects of the study comply with the Declaration of Helsinki. The Ethics Committee specifically approved that no informed consent was required because this was a retrospective study and the data were analyzed anonymously.

Consent for publication

Not applicable. No personal data were collected in the context of this study.

Data availability statement

The data analyzed in the current study can be available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Liping Wang analyzed and interpreted the patient data and performed manuscript edit; Mingjia Dai and Chunyang Li were involved in data collection; Jungui Hao, Fang Ji and Xuebing Yan participated in designing the study, preparation and editing the manuscript. All authors have read and approved the final manuscript.

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