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Original Article

Age and e antigen status are associated with pre-existing tolerance mutations in Chinese patients with chronic HBV infection: a retrospective case control study

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Abstract: Background: The aim of this study was to investigate prevalent mutations of RT region of hepatitis B virus (HBV) genome in NA naïve Chinese patients with chronic HBV infection. Methods: A total of 217 patients with chronic HBV infection (172 with chronic hepatitis B and 45 with liver cirrhosis) were enrolled in this study. Mutations were detected by the direct sequencing of RT region after PCR amplification. Mutations and subsequent amino acid changes were analyzed for 16 well characterized NAs-resistance mutations (rtL180M, rtA181T/V/S, rtT184A/G/I/S, rtS202G/I, rtM204V/I/S, rtN236T and rtM250V/L) and 10 secondary or compensatory resistance mutations (rtV173L, rtV207I/L/G, rtS213T, rtV214A, rtQ215S, rtP237H and rtN/H238T/D). Results: Out of the 217 patients, 151 (69.6%) were male and 67 (30.4%) were female. A total of 166 patients (76.5%) were HBeAg positive. Mutations in the RT region were found in 28 patients (12.9%) including 34 mutations. Primary resistance mutations of HBV RT region were found in 8 (3.7%) cases. Of these 8 patients, 6 had rtM204I including 2 with combination of rtL180M, 1 had rtA181T and 1 had rtT184S substitution. While secondary/compensatory resistance mutations were detected in 20 (9.2%) cases. The most frequent secondary resistance mutation was rtS213T (5.0%), followed by rtV214A/E/P (2.3%) and rtV207L/M/I (1.8%). Multivariate analysis showed that age (P=0.021) and HBeAg status (P<0.001) were associated with HBV resistance mutations, while sex, ALT, AST, TBIL, HBV DNA level, genotype and liver disease stages were not statistically significant. Conclusions: Primary resistance mutations can be detected in treatment naive HBV infected patients, especially in older and HBeAg negative ones. Antivirals with high genetic barriers should be selected to avoid potential treatment failure.

Keywords: HBV, resistance mutation, e antigen status, RT region

Introduction

HBV infection is still a serious world health problem which can lead to liver cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. Recently, approved HBV therapies include interferon alfa (IFN-a) and nucleos(t)ide analogues (NAs) including Lamivudine (LAM), telbivudine (LdT), adefovir (ADV), entecavir (ETV), tenofovir (TDF) and tenofovir alafenamide (TAF) [3-5]. NAs have favorable effects and relatively few side effects, although prolonged NA treatment may result in drug-resistant HBV mutants [6], especially for those with low gene barriers such as lamivudine. Of note, resistance mutations can spontaneously occur within HBV RT region in some NA naive patients, as reported in a few studies [7, 8]. HBV resistant mutants may pre-exist as minor viral population and gradually evolve to predominant population resulting in treatment failure under continuous NA therapy [9].

However, the clinical features associated with the pre-existing resistance mutation in endemic areas have not been well evaluated yet. The aim of the present study was to evaluate the mutations occurring naturally within HBV RT region among NAs naïve Chinese patients with chronic HBV infection, and to investigate the related factors and their clinical implications.

Methods

Patients

A total of 217 inpatients were recruited from August 2009 to July 2016 in the Second Hospital of Shandong University. All the patients had positive test results for hepatitis B surface antigen (HBsAg) for more than 6 months and elevated liver enzymes, including 172 chronic hepatitis B (CHB) and 45 liver cirrhosis cases. None of the patients had received NA treatment prior to the serum samples being collected. Exclusion criteria included hepatitis C virus or human immunodeficiency virus co-infection, autoimmune liver disease, and alcohol or drug abuse. The study protocol was approved by the Ethics Committee of the Second Hospital of Shandong University in accordance with the Declaration of Helsinki, and written consent forms were obtained from every patient enrolled.

Liver biochemistry, HBV serology and HBV DNA assays

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Hitachi Automatic Clinical Analyzer 7170 (Hitachi High-Technologies, Tokyo, Japan). HBV serological markers were determined by chemiluminescent microparticle immunoassay using the Abbot Architect immunoassay system (Abbott Laboratories, AbbottPark, Illinois, USA). HBV DNA was measured by real-time PCR using suitable reagents (Sinomd Gene, Beijing, China) with the lowest detection limit of 500 copies/mL.

HBV DNA extraction, amplification and sequencing

HBV genomes we extracted from 200 μL serum samples using Virus DNA/RNA Extracting Kit (Magnetic beads adsorption) (Sinomd Gene, Beijing, China) according to the manufacturer's instructions. The PCR primers were 5'-ACCTCTAGGTTTCCCTCTTGTTGC-3' (forward, nt 545-568) and 5'-CCACAATTCTTTGACATACTTTCC-3' (reverse, nt 1003-980).

Amplification was performed with Pfu PCR MasterMix (Tiangen Biotech, Beijing, China), 5 μL HBV DNA template and 0.5 μL primer each under the following conditions: initial 95°C denaturation for 3 min, then 95°C denaturation for 15 s, 60°C annealing for 45 s and 65°C extension for 1 min, for a total of 35 cycles.

Then, 5 uL product was mixed with 2 uL of SAP enzyme for enzymolysis. The conditions for enzymolysis were as follows: 37°C for 60 min, 80°C for 15 min. The sequencing PCR was carried out in 6 μL volume, including 31 μL of enzymolysis product, 1 uL of Bigdye and buffer and 1 μL of sequencing primer. PCR conditions for sequencing PCR were as follows: initial denaturation at 96°C for 1 min, 30 cycles consisting of 96°C for 10 sec, 50°C for 5 sec and 60°C for 2 min, finally 65°C for 1 min. The ultimate PCR fragment was purified and sequenced with an ABI 3130 automated sequencer (Applied Biosystems, Foster City, California, USA) using primer 5'-GCACTTGTATTCCCATCCCATCA-3').

HBV genotyping and mutation analysis

In this study, 16 well-characterized mutations associated with NAs-resistance (rtL180M, rtA181T/V/S, rtT184A/G/I/S, rtS202G/I, rtM204V/I/S, rtN236T and rtM250V/L) and 10 secondary or compensatory resistance mutations associated with reduction of susceptibility to NAs (rtV173L, rtV207I/L/G, rtS213T, rtV214A, rtQ215S, rtP237H and rtN/H238T/D) were analyzed [9-13]. Nucleotide and amino acid variations within RT regions were compared with reference HBV sequences of the same genotypes from GenBank, the accession numbers of reference sequences are X02763, X51970, AF090842, D00329, AB073846, AB602818, X04615, M12906, AB014381, X65259, M32138, X85254, X75657, AB032431, X69798, AB036910, AF223965, AF160501, AB064310, AF405706, AY090454, AY090457 and AY090460 [14]. HBV genotypes and nucleotide and amino acid variations were analyzed using the HBVSTAR program online [15].

Statistical analysis

Results were reported as median and range for continuous variables and percentages for categorical variables. The student *t*-test was used to evaluate normally distributed continuous variables, and x^2 test was used to evaluate categorical variables. The Mann-Whitney *U* test was applied to nonparametric variables. A two-tailed *p*-value <0.05 was considered statistically significant. Binary logistic regression was

Table 1. Baseline characteristics of the study population

	HBeAg-positive (n=166)	HBeAg-negative (n=51)	*p* value
Gender (male/female)	111/55	40/11	0.127
Age (years), median (range)	34 (17-73)	46 (11-82)	<0.001
ALT (IU/L), median (range)	158 (15-2450)	153 (24-1115)	0.233
AST (IU/L), median (range)	107 (25-1852)	132 (33-697)	0.854
TBIL (μmol/L), median (range)	20.1 (2.5-418.2)	25 (6.9-450)	0.400
HBV DNA (Log_{10} copies/mL), median (range)	6.7 (2.4-9)	5.85 (2.4-9.3)	<0.001
Genotype (B/C)	3/163	1/50	1.0
Disease stage (Liver cirrhosis/chronic hepatitis B)	26/140	19/32	0.001

Table 2. Observed reverse transcriptase mutations

No.	Age	HBeAg	Mutations	Genotype	No.	Age	HBeAg	Mutations	Genotype
15	37	+	S213T	C	114	51	+	V214A	C
31	30	-	V207I	C	127	66	+	M204I	C
40	44	-	S213T	C	135	42	+	V214P	C
53	40	-	S213T, Q215S	C	146	34	-	S213T	C
55	32	+	A181T	C	147	60	-	M204I	C
62	36	+	N/H238S	C	153	37	-	V207L	C
69	33	+	S213T	C	154	32	+	V207L	C
80	49	-	V214E	C	161	60	-	L180M, M204I	C
82	62	-	S213T	B	162	53	-	M204I	C
86	69	+	L180M, M204I	C	163	45	-	S213T, V214A, Q215E	C
91	38	-	S213T	C	173	36	+	V214A	C
95	50	-	S213T, V207M	C	180	41	-	S213T	C
107	26	+	T184S	C	193	49	+	N/H238T/D	C
112	36	+	M204I	C	207	26	-	S213T	C

+: positive, -: negative.

performed to explore the predictor of resistance mutations.

Results

Patient characteristics

A total of 217 NAs naive patients with chronic HBV infection were enrolled in this study. 151 (69.6%) were male and 67 (30.4%) were female with a median age of 36 years old (11-82). The median HBV DNA level was 6.5 log_{10} copies/mL (2.4-9.3 log_{10} copies/mL). A total of 166 patients (76.5%) were HBeAg positive. The main characteristics of the HBeAg-positive and HBeAg-negative patients were compared (**Table 1**). The table indicated that HBeAg-negative patients were significantly older (P<0.001), and had significantly lower HBV DNA level (P<0.001), but had more advanced liver diseases than HBeAg-positive ones (P=0.001).

Analysis of potential resistance mutations

Out of the 217 patients, resistance related mutations in the RT region were found in 28 patients (**Table 2**; **Figure 1**) including 34 mutations. Primary resistance mutations of HBV RT region were found in 8 (3.7%) patients. Of these 8 patients, 6 had rtM204I substitution and 2 of them had rtL180M simultaneously, 1 had rtA181T and 1 had rtT184S substitution. While secondary/compensatory resistance mutations were detected in 20 (9.2%) patients. The most frequent secondary resistance mutation was found at position rtS213T (11/217, 5.0%). The rtV214 mutations were detected in 5 (2.3%) patients, which included rtV214A mutation in 3 patients, rtV214E mutation in 1 patient and rtV214P mutation in 1 patient. The rtV207 mutations were detected in 4 (1.8%) patients, which included rtV207L mutation in 2 patients, rtV207M mutation in 1 patient and rtV207I

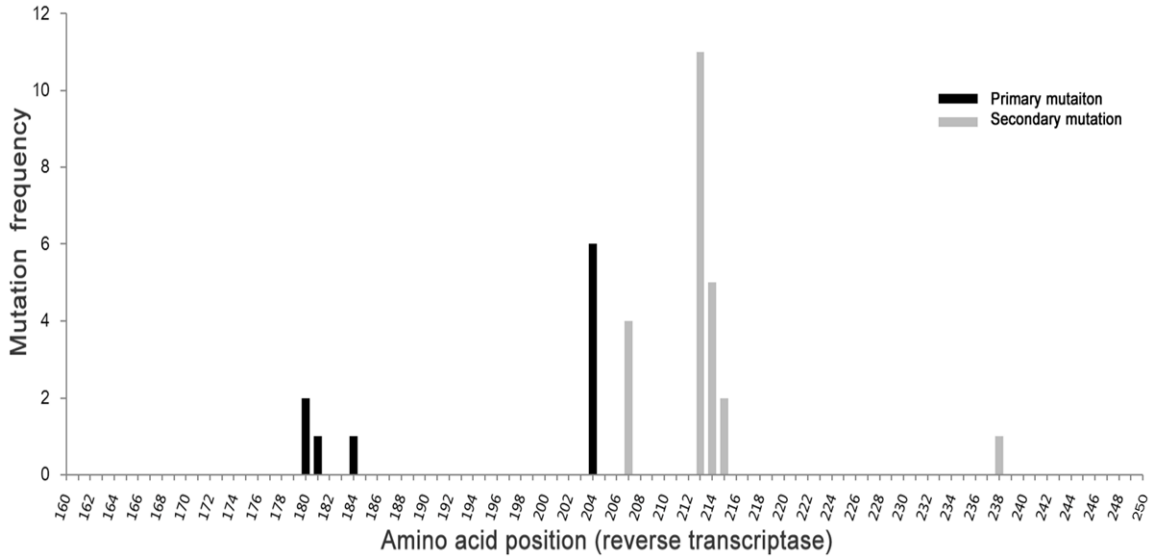

Figure 1. Distribution and frequency of resistance mutation related to nucleos(t)ide analogues in HBV reverse transcriptase region.

mutation in 1 patient. Other common secondary resistance mutations included rtQ215S/E and N/H238T/D/S, which were present in 2 (0.1%) patients each. Of the 28 patients with mutations, 23 (82.14%) had single mutation while 5 (17.86%) had multiple mutations, including 4 with double mutations and 1 with triple mutations.

Factors associated with HBV mutations

To explore the factors associated with the emergence of resistance mutation, clinical features of patients with mutations were compared with those of patients without mutations (**Table 3**). In brief, age and HBeAg status were statistically different between the two groups (P=0.019 and <0.001, respectively), indicating the resistance mutations were detected more frequently in older and HBeAg-negative patients. Other parameters, such as ALT, gender, genotype and HBV DNA level were comparable between groups. Spearman correlation analysis revealed that mutation was related to age and HBeAg status (P<0.01). Logistic regression was performed to investigate the factors related to mutation in multivariate setting (**Table 4**). HBeAg status and age >30 y were predictors for pre-existing mutations, (P<0.05).

Discussion

In the present study, HBV RT sequences covering all well-characterized resistance mutations were determined in 217 patients with chronic HBV infection. Thirty five resistance mutations were detected in 28 patients (12.9%), among which primary resistance mutations were found in 8 patients (3.7%). The most frequent primary mutation detected was rtM204I, and the most frequent compensatory mutation detected was rtS213T. Correlation analysis revealed that HBeAg status and age were associated with resistance mutation, and logistic regression indicated that HBeAg negativity and age >30 years were risk factor for pre-existing resistance mutations.

Owing to a lack of proofreading capacity during reverse transcription and a high replication rate, HBV mutations can arise rapidly. Resistance mutations in HBV polymerase associated with NAs could be classified into primary and secondary (or compensatory) mutation. Primary resistance mutations contain an amino acid substitution that significantly decrease susceptibility to an antiviral NA, while secondary resistance mutations have no direct role but restore functional defects of HBV mutant containing primary mutations and enhance resistance to antiviral agents [11]. In our study, primary resistance mutations of HBV RT region were found in 3.7% (8/217) cases. Of these 8 patients, 6 had rtM204I, 1 had rtA181T and 1 had rtT184S substitution. The rtM204I/V substitutions are primary resistance mutations associated with antiviral resistance to LAM and LDT. The rtM204I substitution has been detect-

Table 3. Clinical characteristics of the study population with mutation and without mutation

Characteristics	With mutation (n=28)	Without mutation (n=189)	*p* value
Gender (male/female)	19/9	131/58	1.000
Age (years), median (range)	41 (26-69)	36 (11-82)	0.019
ALT (IU/L), median (range)	142 (24-822)	159 (15-2450)	0.431
AST (IU/L), median (range)	113 (33-656)	116 (25-1852)	0.308
TBIL (umol/L), median (range)	27.5 (6.9-310.5)	20.3 (2.5-450)	0.193
HBV DNA (Log_{10} copies/mL), median (range)	6.3 (2.7-8)	6.6 (2.4-9.3)	0.208
Genotype (B/C)	1/27	3/186	0.427
HBeAg status (+/-)	13/15	153/36	<0.001
Disease stage (Liver cirrhosis/chronic hepatitis B)	9/19	36/153	0.111

+: positive, -: negative.

Table 4. Logistic analysis of factors associated with pre-existing mutations

	Univariate			Multivariate		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Gender	1.070	0.457-2.506	0.876			
Age >30 years	5.244	1.529-17.99	0.008	4.692	1.263-17.428	0.021
ALT	0.999	0.998-1.001	0.469	1.003	1.000-1.007	0.070
AST	0.999	0.996-1.001	0.273	0.994	0.989-1.000	0.057
TBIL	1.007	0.997-1.007	0.457			
HBV DNA	0.823	0.608-1.115	0.209			
Genotype	0.435	0.044-4.339	0.478			
HBeAg status	4.904	2.145-11.209	<0.001	4.429	1.846-10.627	<0.001
Disease stage	2.013	0.842-4.816	0.116			

ed in isolation, but rtM204V mutation is usually associated with the compensatory rtL180M mutation. In our study, only rtM204I substitution were detected, and most of them are alone, which was consistent with previous studies [13, 16].

Many studies have shown that mutations occur spontaneously in YMDD motif of HBV RT region among NA untreated patients. The prevalence of resistance mutation in NA-naïve patients seemed contradictory. Villa et al reported no YMDD mutant (0%) among LAM-untreated patients [17], while Wang et al determined 31.7% for incidence of YMDD motif mutation among NA naive patients [18]. Also, in a recent meta-analysis, Tan et al reported that overall incidence of spontaneously occurring YMDD mutation in LAM-untreated patients was up to 12.21%, and China had an incidence of 13.38% [16]. The various prevalence of YMDD mutant may partially result from the different methods adopted to detect the mutation. The methods such as the line probe assay and restriction fragment length polymorphism (RFLP), are more sensitive than direct PCR sequencing (5% vs. 20%), thus the prevalence of mutants detected by more sensitive methods seems higher [9, 11]. On the other hand, the enrollment of patients played an important role since the NA-experienced patients have a much higher prevalence of resistance mutation. Our study showed a lower prevalence in NA naive patients with chronic HBV infection. The rtM204I had been detected in only 2.76% (6/217) patients, which may reflect the prevalence of rtM204 in NA naive patients in patients with genotype C in endemic area. This prevalence was similar with the study performed by Han, et al from China, which reported that the prevalence of YMDD mutants was 1.8% [7].

HBeAg negativity was found as risk factor for pre-existing resistance mutations. HBeAg-negative CHB represents a later immune reactive phase in the natural history of chronic HBV infection, which may follow HBeAg seroconversion during the immune reactive phase or may develop after years or decades of the inactive carrier state [3, 5]. HBV exists as quasispecies, which consists of a spectrum of mutants which are genetically similar but not identical [19, 20]. HBeAg seroconversion means an extra immune selective pressure for HBV quasispecies, which

can accelerate the HBV quasispecies evolution and generated more mutations than those without HBeAg seroconversion. Additionally, patients with HBeAg-negative CHB harbor a predominance of HBV virions with mutations in the precore and/or the basal core promoter regions. Those mutations enable the virons not to express or express low levels of HBeAg, although they can enhance the replication capacity as compensation [21]. Therefore, more mutations can be generated due to a higher replication capacity. Finally, patients with HBeAg-negative CHB usually experience periodic reactivation with fluctuating aminotransferases levels and active hepatitis; the inflammations merely reflect the immune activity that means another kind of selective pressure driving the HBV evolution.

Age is another risk factor for tolerance mutation. That is, tolerance mutations occur more frequently in older patients. As most chronic HBV infection was acquired perinatally in China, longer infection duration results in more cumulative mutations. Although HBeAg negativity is more popular in older patients, no interaction between HBeAg and age was found in multivariate analysis (data not shown). Subgroup analysis showed that age >30 years was predictor of resistance mutation both in HBeAg-positive patients and in HBeAg-negative patients.

Although primary resistance mutations related to lamivudine, telbivudine and adefovir were detected in our study, the triple mutation related to entecavir was not found. Adefovir dipivoxil and lamivudine are not recommended as the first-line choice for CHB treatment by the practice guidelines from EASL, AASLD and APASL, although they are still widely used in China because of their good affordability and availability. Our data showed that the pretreatment resistance mutation related with adefovir and lamivudine do exist and may cause treatment failure in early stage. As pretreatment mutation may result in treatment failure in CHB patients [22], lamivudine and adefovir should not be selected as the initiation antiviral drug, especially in older and HBeAg-negative CHB patients.

Conclusions

Our study showed that primary resistance mutations can be detected in treatment naive HBV infected patients, and rtM204I is the most frequent. Age and HBeAg status were associated with the occurrence of resistance mutations. Antivirals with high genetic barriers should be selected to avoid potential treatment failure, especially in older and HBeAg negative CHB patients.

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Disclosure of conflict of interest

None.

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