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Brief Original Article

Hepatitis B virus pre-existing drug resistant mutation is related to the genotype and disease progression

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Abstract

Introduction: Previous studies have indicated that the drug-resistant mutations of hepatitis B virus (HBV) are a major obstacle to antiviral therapy. However, it is still unclear whether there are pre-existent resistance mutations in patients with HBV infection and the relationship between drug-resistant mutation, genotypes, and progression of hepatitis B disease.

Methodology: A total of 357 treatment-naïve patients with HBV infection were involved in this retrospective study. The drug-resistant mutations of HBV reverse transcriptase domain were screened by direct gene sequencing.

Results: Lamivudine (LAM) resistance was detected in 8 patients (3.7%) with chronic hepatitis B (CHB), 13 (11.7%) patients with liver cirrhosis (LC), and 6 (21.4%) patients with hepatocellular carcinoma (HCC). Adefovir(ADV)-resistant mutations were detected in 10 (4.6%) patients with CHB, 15 (13.5%) patients with LC and 4 (14.5%) patients with HCC. Both LAM and ADV resistant mutations were detected in 2 patients (0.9%) with CHB, 1 patient (0.9%) with LC and 1 patient (3.6%) with HCC. Significant differences ($p < 0.01$) were observed in the drug-resistance rates among patients with CHB, LC and HCC. Meanwhile, all the drug-resistant mutations were found in patients with HBV genotype C.

Conclusions: This study demonstrated higher risk of pre-existing drug-resistant mutations in patients with HBV genotype C comparing to patients with HBV genotype B. Likewise, increasing prevalence of pre-existing drug-resistant mutations was shown, alongside with the progression of the disease.

Key words: hepatitis B virus; pre-existing drug resistance; mutation; reverse transcriptase.

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Introduction

Antiviral treatment is an effective way to inhibit hepatitis B virus (HBV) replication and to delay the progress of the disease. The nucleos(t)ide analogues (NAs) are extensively used in clinical work. However, the efficacy of NAs like including lamivudine (LAM), adefovir (ADV), telbivudine (LDT) are challenged during the treatment by drug resistance [1-3]. The resistance rate of entecavir (ETV) is relatively low, five-years drug resistance rate is about 1.2% [4]. However, the resistance rate of ETV will be up to 51% in five years in patients with LDT resistance [5]. And virologic breakthrough was also reported in patients with LAM resistance and co-infected with human immunodeficiency virus (HIV) during treatment with tenofovir disoproxil fumarate (TDF) [6].

Therefore, the presence of drug resistant strains has brought great difficulties to the treatment of HBV. Worldwide guidelines currently recommend TDF-based monotherapy or combination therapy as the first-line treatment for patients with antiviral-resistant chronic hepatitis B (CHB) [7-9] although TDF is not large-scale used in Northern China because of its elevated cost. For most patients, LAM or ADV are still selected. As the NAs are inhibitors of RT domain of HBV polymerase, due to the absence of proofreading activity, the HBV polymerases/RT leads to the introduction of random mutations into HBV genome. The error rate of HBV polymerase is approximately of 1×10^5 to 10^7 base syntheses, as result of the highly error-prone nature of the HBV reverse transcriptase (RT) [10,11]. Under the selective pressure by means of the administration of antiviral agents, quasi species of HBV converge on a dominant HBV mutant that escapes selection pressure, creating a drug-resistant HBV strain. The question is whether the drug-resistant strains are preexistent or drug induced. There is also primary non-response phenomenon in the course of antiviral therapy. There are doubts on pre-existent resistance mutations in patients without NAs therapy. Therefore, based on our

results a HBV resistance mutation detection was performed and selected effective antiviral drugs were administered. In the present study, we further investigated the clinical characteristics of HBV infection, HBV genotype distribution, and HBV pre-existent resistance mutations in CHB patients.

Methodology

Patients

In total, 357 patients with CHB (HBV DNA positive) were included in the study. All patients were treated in the Department of Infectious Diseases, Affiliated Hospital of Xuzhou Medical University, between April 2011 and April 2014. The samples were collected from patients with positive HBsAg for more than six months, and with twice more than the normal level of alanine aminotransferase (ALT). Liver cirrhosis was diagnosed by liver function tests and ultrasonography (US) or Computerized Tomography (CT). The diagnosis of hepatocellular carcinoma (HCC) was based on US, CT, elevated level of serum fetoprotein (AFP≥200ng/mL), or by needle aspiration biopsy of liver (for samples with low AFP level). No patients had anamnestic data about treatment with NAs or interferon. Exclusion conditions were: overlapping infection of hepatitis A, C, D or E; Epstein barr virus, HIV infection; cytomegalovirus infection; combining with alcoholic liver disease or autoimmune diseases.

The study protocol was approved by the local Research and Ethics Committee at Affiliated Hospital of Xuzhou Medical University, in accordance with the guidelines of the 1975 Declaration of Helsinki. All participants gave written informed consents.

Instruments and reagents

The used methodology include 7500 real-time PCR system (Applied Biosystems, Darmstadt, Germany) and 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA), HBV RT region (covered B domain to E domain) was amplified using 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 PCR amplification and sequencing reagents (Shenyou Biological Engineering Company, Shanghai, China).

HBV DNA sequencing and analysis of resistant mutations

HBV DNA template was prepared following the protocol of extraction kit (HBV and Drug Resistance Related Mutation Detection Kit (Shenyou Biological Engineering Company, Shanghai, China.). 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 webside).

Statistical analysis

SPSS16.0 software (SPSS, Chicago, 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. P value ≤0.05 was considered statistically significant difference.

Results

Characteristics of enrolled patients

The general information on 357 patients is shown in the Table 1. There was no significant difference in the sex, and ratio of HBV genotype among three groups divided by clinical status ($p > 0.05$). However, the HBeAg positive ratios were different in 3 groups ($p < 0.01$) (Table 2). HBV genotypes C (336/357; 94.1%) and B (21/357; 5.9%) are predominant in Xuzhou, Jiangsu Province, while HBV genotypes A, D, E, F, G, and H were not detected in this study.

Table 1. Characteristics of enrolled patients.

Items	Values n (%)
Age in years (mean ± SD)	40.69 ± 13.63
Gender	
Males	276（77.3）
Females	81 (22.7)
Genotype	
Type B	21（5.9）
Type C	336（94.1）
No.(%) of HBeAg positive patients	224（62.7）
Clinical status	
CHB (%)	218（61.1）
LC (%)	111（31.1）
HCC (%)	28（7.8）
Serum HBV DNA (log10IU/ml)	6.31±1.35

CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

Table 2: Genotype and HBeAg in different diagnose patients.

	Type B		Type C		Total	
	HBeAg +	HBeAg -	HBeAg +	HBeAg -	HBeAg +	HBeAg -
CHB (n = 218)	11 (73.3%)	4 (26.7%)	147 (72.4%)	56 (27.6%)	158 (72.5%)	60 (27.5%)
LC (n = 111)	2 (33.3%)	4 (66.7%)	54 (51.4%)	51 (48.6%)	56 (50.5%)	55 (49.5%)
LCC (n = 28)	0 (-)	0 (-)	10 (35.7%)	18 (64.3%)	10 (35.7%)	18 (62.7%)

CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; LC, liver cirrhosis; HBeAg positive ratios were analyzed by chi-squared test among three groups, $\chi^2 = 24.763$, $p < 0.001$; The distribution of HBV genotype among three groups were analyzed by chi-squared test, $\chi^2 = 1.950$, $p = 0.377$.

Table 3. Pre-existing resistant mutations in different diagnose groups.

Group	LAM*	ADV*	LAM & ADV	Total
CHB (n = 218)	8 (3.7%)	10 (4.6%)	2 (0.9%)	20（9.2%）
LC (n = 111)	13 (11.7%)	15 (13.5%)	1 (0.9%)	29 （26.1%）
LCC (n = 28)	6 (21.4%)	4 (14.3%)	1 (3.6%)	11 （39.3%）
Total	27（7.6%）	29（8.1%）	4（1.1%）	60（16.8%）
χ^2	15.159	9.398	1.648	
p-value	0.001	0.009	0.439	

CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; LC, liver cirrhosis; * Ratios of different NA related mutations in three group were analyzed by chi-squared test.

Table 4. Prevalence of hepatitis B virus genotype and resistant mutations in the study cohort.

Genotype		mutation type	CHB	LC	HCC	Resistant drug
B	C					
0	2	rtV173L	0	2	0	LAM
0	1	rtL180M	0	0	1	LAM/LDT
0	2	rtA181T/V	0	1	1	ADV
0	1	rtM204I/V/S	0	1	0	LAM/LDT
0	4	rtV207I/L/G	2	1	1	LAM
0	17	rtS213T	6	7	4	LAM
0	13	rtV214A	4	7	2	ADV
0	2	rtQ215S	0	2	0	ADV
0	11	rtN/H238T/D	6	4	1	ADV
0	11	rtL180M+rtS213T	0	1	0	LAM
0	1	rtA181T/V+rtV214A	0	1	0	ADV
0	1	rtV207I/L/G+ rtS213T	0	1	0	LAM
0	1	rtV207I/L/G+ rtV214A	0	0	1	LAM+ADV
0	1	rtV207I/L/G+ rtN/H238T/D	1	0	0	LAM+ADV
0	1	rtS213T+ rtN/H238T/D	1	1	0	LAM+ADV
0	28	191V/I	15	10	3	Unknown
0	10	224I/V	4	3	3	Unknown

Pre-existing resistant mutations and the progression of the disease.

Because LDT has the overlapped mutation sites with LAM, therefore we only analyzed mutations which resist against LAM and ADV. The results showed that the ratios of ADV resistance mutations were different in different state of the disease,and with the progression of the disease the ratios of ADV resistance mutations increased ($P < 0.01$), The same was found in the ratio of LAM resistant mutations ($p < 0.01$). There were 4 cases simultaneously with mutations both against LAM and ADV (Table 3).

The relationship between HBV genotype and pre-existing resistant mutations

Out of all patients, 16.8% (60/357) were found with known resistance mutations in HBV RT domain. All mutations occurred in patients with HBV genotype C, and no resistance mutation was found in patients with genotype B. The resistant mutation modes were complicated; they included known site such as rtV173L, rtL180M, rtA181V/T/S, rtM204I/V/S, rtV207I/L/G, rtS213T, rtV214A, rtQ215S, rtN/H238T/D and other multi-drug resistance mutation. We found that main mutations occured at the site were: S213T (17/357, 4.8%), rtV214A (13/357, 3.6%), rtN/H238T/D (11/357, 3.1%) and rtL180M+rtS213T (11/357, 3.1%) in NAs therapy naive patients. These mutations can lead to the resistance to LAM or ADV. There were two patients who had rtA181T/V mutation, which meant they are resistant against LAM, ADV and LDT. There were no ETV and TDF pre-existing resistance mutation in our study. There were two unknown mutation sites rtV191/I (28/337,7.8%) and rt224I/V (10/357, 2.8%) (Table 4).

Discussion

In the present study, 357 HBV infected patients without NAs treatment were recruited. Drug resistant mutations in HBV RT domain were detected by direct sequencing. Furthermore, we found that there were LAM and ADV preexisting resistance in patients of HCC, liver cirrhosis and CHB. The rates of LAM and ADV resistance became higher with disease progress. The resistant mutation modes were complicated, they included known site such as rtV173L, rtL180M, rtA181V/T/S, rtM204I/V/S, rtV207I/L/G, rtS213T, rtV214A, rtQ215S, rtN/H238T/D and other multi-drug resistance mutations. We found no ETV and TDF preexisting resistance mutation. According to our data, HBV genotype B and C were the main strains in this group. All the pre-existing resistance mutations were found only in genotype C, but not in genotype B. This indicated HBV preexisting resistance mutation in genotype C is more common than genotype B. In this study, we found mutations as rtS213T (4.8%), rtV214A (3.6%), rtQ215S (0.6%), rtN/H238T/D (3.1%) in NAs therapy naive patients.

Mutations in the RT region of the polymerase gene at amino acid location rtA181T/V, rtM204V/I/S, rtN236T, and rtM250I/V were interpreted as primary resistance mutations, while rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184A/C/G/S, rtS202C/G/I , rt214, rtQ215S , rtL217P, rtL229M, rtI233V and rtN238H were considered as secondary or compensatory mutations [12-14]. Rt214, rt215, rt221 and rt238 mutations could reduce the antiviral efficacy of ADV, and was considered as “minor mutations” or “propose mutations” [3,15,16]. From the study, we found that primary resistance mutations (rtA181T/V, rtM204V/I/S, rtN236T, and rtM250I/V) had low prevalence , while compensatory mutations such as rtV173L, rtV214A, rtQ215S, and rt N/H238T/D had high prevalence.

Given the lower prevalence of HBV drug-resistant in the naïve patients, some investigators suggested that routine testing for resistant mutation before initiating antiviral therapy is not necessary [13,17] But further research has revealed that tyrosine-methionine-aspartate-aspartate (YMDD) mutations also exist in patients with CHB infection without LAM treatment [18,19], Zhao Y *et al.* [20] used INNO-LiPA assay to detect mutations in HBV DNA polymerase associated with NAs resistance in 269 treatment-naive patients with CHB and found 24 patients (8.9%) with detected mutations in HBV DNA polymerase. In this study we found 60 patients (16.8%) with pre-existing resistant mutations in HBV chronically infected patients. Along with severity of the disease, there was an increasing tendency of prevalence of resistant mutations. So we thought it was better to perform a screening of drug resistance mutations before initiation of the treatment with NAs, especially for patients with LC or HCC .

In this study, the common mutation sites of LAM or ADV, such as rtV173L, rtL180M, rtA181V/T/S, rtM204I/V/S, rtV207I/L/G, rtS213T, rtV214A, rtQ215S, rtN/H238T/D were found, and other two mutation sites which were rtV191/I (7.8%) and rt224I/V (2.8%). AminiBavil-Olyaee [21] in 2009 revealed HBV rtV191I mutation in HIV-HBV co-

infected and HBsAg-negative patients, during TDF therapy, and found it was resistant to LAM, but not TDF. Therefore, rtV191I mutation may be the new resistance site to LAM. Since rt224I/V has never been reported before [13,22-26], further observation of these patients might address the question whether these mutations are associated with other nucleoside analogue resistance or affect the outcome of the disease.

This study has several limitations. First, direct PCR sequencing is considered to be the gold standard for detecting genotypic resistance mutations, but it is not the most senstive. The population-based sequencing sensitivity often decreases when it is used to detect mutants less than 20% in ratio. Second, cases with low HBV qualification would not be identified.

Conclusions

This study indicated that the pre-existing antiviral resistance occurs in chronic HBV infected patients. The results may provide a novel insight into the relationships between clinical severity, HBV genotype distribution, and HBV naturally occurring variants in these Chinese patients. Along with the severity of the disease, there was an increasing tendency of prevalence of pre-existing drug-resistant mutations in the chronic patients, and genotype C is proner to develop resistant mutations than genotype B. With regard to the high rate of resistant mutation in LC or HCC patients, ETV or TDF should be recommended to use ,LAM or ADV monotherapy or combined use is not the optimal selection.

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References

1. Morgan M, Keeffe EB (2009) Diagnosis and treatment of chronic hepatitis B: 2009 update. Minerva Gastroenterol Dietol 55: 5-22.
2. Nguyen MH, Keeffe EB (2009) Chronic hepatitis B: early viral suppression and long-term outcomes of therapy with oral nucleos(t)ides. J Viral Hepat 16: 149-155.
3. Deng L, Tang H (2011) Hepatitis B virus drug resistance to current nucleos(t)ide analogs: Mechanisms and mutation sites. Hepatol Res 41: 1017-1024.
4. Chang TT, Lai CL, Kew YS, Lee SS, Coelho HS, Carrilho FJ, Poordad F,Halota W, Horsmans Y, Tsai N, Zhang H, Tenney DJ, Tamez R, Iloeje U (2010) Entecavir treatment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. Hepatology 51: 422-430.
5. Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, Wichroski MJ, Xu D, Yang J, Wilber RB, Colonno RJ (2009) Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. Hepatology 49: 1503-1514.
6. Sheldon J, Camino N, Rodés B, Bartholomeusz A, Kuiper M, Tacke F, Núñez M, Mauss S, Lutz T, Klausen G, Locarnini S, Soriano V (2005) Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. Antivir Ther 10: 727-734.
7. EASL clinical practice guidelines (2012) Management of chronic hepatitis B virus infection. J Hepatol 57: 167-185.
8. KASL Clinical Practice Guidelines (2012) Management of chronic hepatitis B. Clin Mol Hepatol 18: 109-162.
9. Lok AS, McMahon BJ (2009) Chronic hepatitis B: update 2009. Hepatology 50: 661-662.
10. Girones R, Miller RH (1989) Mutation rate of the hepadnavirus genome. Virology. 170: 595-597.
11. Kim JH, Park YK, Park ES, Kim KH (2014) Molecular diagnosis and treatment of drug-resistant hepatitis B virus. World J Gastroenterol 20: 5708-5720.
12. Mantovani N, Cicero M, Santana LC, Silveira C, do Carmo EP, Abrao PR, Diaz RS, Caseiro MM, Komninakis SV (2013) Detection of lamivudine-resistant variants and mutations related to reduced antigenicity of HBsAg in individuals from the cities of Santos and Sao Paulo, Brazil. Virol J 10: 320.
13. Vutien P, Trinh HN, Garcia RT, Nguyen HA, Levitt BS, Nguyen K, da Silveira E, Daugherty T, Ahmed A, Garcia G, Lutchman GA, Nguyen MH (2014) Mutations in HBV DNA polymerase associated with nucleos(t)ide resistance are rare in treatment-naive patients. Clin Gastroenterol Hepatol. 12: 1363-1370.
14. Bartholomeusz A, Locarnini SA (2006) Antiviral drug resistance: clinical consequences and molecular aspects. Semin Liver Dis. 26: 162-170.
15. Salpini R, Svicher V, Cento V, Gori C, Bertoli A, Scopelliti F, Micheli V, Cappiello T, Spanò A, Rizzardini G, De Sanctis GM, Sarrecchia C, Angelico M, Perno CF (2011) Characterization of drug-resistance mutations in HBV D-genotype chronically infected patients, naïve to antiviral drugs. Antiviral Res 92: 382-385.
16. Santantonio T, Fasano M, Durantel S, Barraud L, Heichen M, Guastadisegni A, Pastore G, Zoulim F (2009) Adefovir dipivoxil resistance patterns in patients with lamivudine-resistant chronic hepatitis B. Antivir Ther 14: 557-565.
17. Panigrahi R, Biswas A, De BK, Chakrabarti S, Chakravarty R (2013) Characterization of antiviral resistance mutations among the Eastern Indian Hepatitis B virus infected population. Virol J 10: 56.
18. Matsuda M, Suzuki F, Suzuki Y, Tsubota A, Akuta N, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Satoh J, Takagi K, Kobayashi M, Ikeda K, Kumada H (2004) Low rate of YMDD motif mutations in polymerase gene of hepatitis B virus in chronically infected patients not treated with lamivudine. J Gastroenterol 39: 34-40.
19. Yang JH, Zhang H, Chen XB, Chen G, Wang X (2013) Relationship between hepatocellular carcinoma and hepatitis B

virus genotype with spontaneous YMDD mutations. World J Gastroenterol 19: 3861-3865.
20. Zhao Y, Wu J, Sun L, Liu G, Li B, Zheng Y, Li X, Tao J (2016) Prevalence of mutations in HBV DNA polymerase gene associated with nucleos(t)ide resistance in treatment-naive patients with Chronic Hepatitis B in Central China. Braz J Infect Dis 20: 173-178.
21. Amini-Bavil-Olyaee S, Herbers U, Mohebbi SR, Sabahi F, Zali MR, Luedde T, Trautwein C, Tacke F (2009) Prevalence, viral replication efficiency and antiviral drug susceptibility of rtQ215 polymerase mutations within the hepatitis B virus genome. J Hepatol 51: 647-654.
22. Fung J, Cheung C, Chan SC, Yuen MF, Chok KS, Sharr W, Dai WC, Chan AC, Cheung TT, Tsang S, Lam B, Lai CL, Lo CM (2011) Entecavir monotherapy is effective in suppressing hepatitis B virus after liver transplantation. Gastroenterology 141: 1212-1219.
23. Mirandola S, Campagnolo D, Bortoletto G, Franceschini L, Marcolongo M, Alberti A (2011) Large-scale survey of naturally occurring HBV polymerase mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B. J Viral Hepat 18: e212-216.
24. Baxa DM, Thekdi AD, Golembieski A, Krishnan PV, Sharif O, Kizy A, Shetron-Rama L, Jovanovich J, Chappell BJ, Snow-Lampart A, Borroto-Esoda K, Gordon SC (2013) Evaluation of anti-HBV drug resistant mutations among patients with acute symptomatic hepatitis B in the United States. J Hepatol 58: 212-216.
25. Lim YS, Yoo BC, Byun KS, Kwon SY, Kim YJ, An J, Lee HC, Lee YS (2016) Tenofovir monotherapy versus tenofovir and entecavir combination therapy in adefovir-resistant chronic hepatitis B patients with multiple drug failure: results of a randomised trial. Gut 65: 1042-1051.
26. Hermans LE, Svicher V, Pas SD, Salpini R, Alvarez M, Ben Ari Z, Boland G, Bruzzone B, Coppola N, Seguin-Devaux C, Dyda T, Garcia F, Kaiser R, Köse S, Krarup H, Lazarevic I, Lunar MM, Maylin S, Micheli V, Mor O, Paraschiv S, Paraskevis D, Poljak M, Puchhammer-Stöckl E, Simon F, Stanojevic M, Stene-Johansen K, Tihic N, Trimoulet P, Verheyen J, Vince A, Weis N, Yalcinkaya T, Lepej SZ, Perno C, Boucher CA, Wensing AM (2016) Combined analysis of the prevalence of drug-resistant hepatitis B virus in antiviral therapy-experienced patients in Europe (CAPRE). J Infect Dis 213: 39-48.

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