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Original Article

Monitoring of genotypic resistance profile in chronic hepatitis B patients receiving nucleos(t)ide analogues in Huzhou, China

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Abstract

Introduction: Antiviral drug-resistance patterns of hepatitis B virus (HBV) mutants are complex and currently partly understood. The aim of this study was to monitor the genotypic resistance profile in patients with chronic hepatitis B (CHB) receiving nucleos(t)ide analogues (NAs) in Huzhou, eastern China.

Methodology: Serum samples of 139 CHB patients undergoing NA treatment were obtained from Huzhou Central Hospital. The full-length HBV reverse transcriptase regions were amplified and sequenced. The NA resistance mutation positions, including rtL80, rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtI233, rtN236, and rtM250 were analyzed.

Results: Genotypic resistance mutations were detected in 41.72% (58/139) of patients with CHB. Drug resistance mutations were detected at positions rt80, rt173, rt180, rt181, rt194, rt202, rt204, rt236, and rt250, but were not observed at positions rt169, rt184, and rt233. The prevalence of mutations at rtM204 was 54.44% in 90 patients who were treated with lamivudine (LAM) or telbivudine (LDT). RtN236 mutations were detected in 7.14% (2/28) of the patients receiving adefovir (ADV) therapy. Additionally, rtA181 mutations were observed in 4 patients with LAM, ADV, and LDT-based therapy, but not in those patients treated with entecavir (ETV). Among patients who harbored rtM204 combination mutations, rtM204I and rtM204V were significantly associated with rtL80I/V and rtL180M, respectively.

Conclusions: The mutation patterns of NA-resistant HBV are complicated in CHB patients in the current clinical setting. Thus, it is necessary to persistently monitor the resistance mutations of HBV for optimizing antiviral therapy strategy and for preventing an outbreak of clinical resistance.

Key words: hepatitis B virus; nucleos(t)ide analogues; resistance; mutation.

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Introduction

Hepatitis B virus (HBV) infection is a worldwide public health problem. It is estimated that about 350 million people worldwide [1] and 93 million people in China [2] are chronic HBV carriers. HBV causes acute and chronic liver diseases, even fatal liver cirrhosis and hepatocellular carcinoma [3].

At present, nucleos(t)ide analogues (NAs) are one type of classic drugs for treatment of HBV infection. Four NAs, including lamivudine (LAM), adefovir (ADV), entecavir (ETV), and telbivudine (LDT), are currently approved in China [4,5]. The target of these NAs is the reverse transcriptase (RT) of the HBV genome. NAs can suppress the replication of HBV by inhibiting the activity of the RT. However, drug resistance is a serious problem caused by long-term antiviral therapy with NAs [6,7]. One or several distinct amino acid mutations in the RT region may reduce the susceptibility of NAs and lead to HBV drug resistance [8,9].

Currently, the genotype resistance mutations are divided into two types, primary resistance mutation and secondary and/or compensatory mutation. The former, which includes mutations of rtA181T/V, rtM204V/I, and rtN236T in the RT region, are responsible for reduced treatment susceptibility of NA therapy [7,10]. The latter, which includes mutations of rtL80I, rtV173L and rtL180M in the RT region could restore the RT activity which defects is caused by primary resistance mutations [11-13]. Therefore, the replication potential of HBV could persist with compensatory mutation.

With the wide application of NAs, the resistance mutation patterns are more and more complex [14,15]. Furthermore, multidrug-resistant (MDR) HBV appears [7]. Hence, it is beneficial to the effective treatment and rescue therapy of HBV infection to understand the

prevalence of genotypic resistance patterns in chronic hepatitis B (CHB) patients.

To date, the incidence and patterns of genotypic resistance mutations have not been extensively investigated in different parts of China. Thus, the purpose of this study was to conduct a hospital-based survey about the genotypic resistance patterns and features in CHB patients receiving NAs in Huzhou, eastern China.

Methodology

Study patients

This was a hospital-based, cross-sectional survey. From September 2011 to December 2013, serum samples were collected from 139 CHB patients in Huzhou Central Hospital (a tertiary hospital) when those samples were sent to the central laboratory for detection of HBV DNA levels. At the time of sampling for HBV genotyping, all patients were HBsAg positive for > six months, and the HBV DNA levels were ≥ 1.0×10^4 copies/mL. All patients were receiving NAs (LAM, ADV, ETV, and LDT) monotherapy in combination or sequential treatment when the serum samples were obtained. The diagnosis of CHB was made according to the Chinese consensus criteria [16]. All patients co-infected with hepatitis C virus, hepatitis D, virus, or human immunodeficiency virus (HIV) were excluded.

Ethical considerations

This study was approved by the ethics committee of Huzhou Central Hospital, and it conformed to the provisions of the Declaration of Helsinki. Written informed consent was obtained from all patients before this study.

Serological markers and quantification of serum HBV DNA levels

Routine serological markers for hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs), hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe), hepatitis B c antibody (anti-HBc), and other biochemical markers were detected in the clinical laboratory of Huzhou Central Hospital. Serum HBV-DNA level was quantified by real-time quantitative polymerase chain reaction (PCR) kits (ZJ Bio-Tech Co., Ltd, Shanghai, China), and the lowest detection limit of this kit was 500 copies/mL.

Amplification of the RT region in the HBV genome

HBV-DNA was extracted from 200 uL serum using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The entire RT region (nt54–1278) of HBV was amplified using nested PCR as previously described [17]. The sequence of the PCR primers is shown in Table 1.

Briefly, outer primers UP3 and DOWN1 were used for the first-round amplification under the following conditions: 10 cycles of 94°C for 35 seconds, 59°C for 35 seconds, 72°C for 70 seconds, followed by 30 cycles of 94°C for 35 seconds, 56°C for 35 seconds, and 72°C for 70 seconds. Inner primers UP4 and DOWN2 were used for second-round PCR; the reaction consisted of 35 cycles of 94°C for 25 seconds, 56°C for 25 seconds, and 72°C for 50 seconds.

DNA sequencing and sequence analysis

The PCR products were purified using a QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany). The bi-directional sequencing was performed by Biowing Applied Biotechnology Co., Ltd. (Shanghai, China) using an ABI Prism 3130X automatic genetic analyzer (Applied Biosystems, Foster City, USA). The primers UP4 and DOWN2 were used for sequencing.

Genotypes of HBV were determined by the genotyping tool of the National Center for Biotechnology Information (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi). The 12AA positions related to drug resistance mutations (Table 2) in the RT region were analyzed according to sequencing results.

Table 1. Amplification primers for reverse transcriptase sequence of the hepatitis B virus genome.

Primer set	Primer name	Sequence(5'-3')	Position(nt)
First round primers			
Sense	UP3	5'-AGT CAG GAA GAC AGC CTA CTC C-3'	nt 3146-3167
Antisense	DOWN1	5'-AGG TGA AGC GAA GTG CAC AC-3'	nt 1577-1596
Second round primers			
Sense	UP4	5'-TTC CTG CTG GTG GCT CCA GTT C-3'	nt 54-75
Antisense	DOWN2	5'-TTC CGC AGT ATG GAT CGG CAG-3'	nt 1258-1278

Statistical analysis

Data were analyzed using SPSS software version 17.0 (IBM, Armonk, USA). Mann-Whitney analysis and student's t test were used to compare quantitative values. Fisher's exact test was used to compare qualitative values. *P* values of < 0.05 were considered to be statistically significant.

Results

Characteristics of the CHB patients

Among 139 CHB patients, the average age was 37.6 years (range: 18–72 years). Of them, 73.38% (102/139) were male and 16.62% (37/139) were female. The HBeAg-positive rate was 47.48% (66/139). There were 53.96% (75/139) patients infected with HBV genotype B, and 46.04% (64/139) patients infected with HBV genotype C; the distribution of genotypes was consistent with a previous report in China [19]. There were no significant differences in sex ratio and distribution of genotypes between the HBeAg-positive and HBeAg-negative patients (all $p > 0.05$). The serum HBV DNA levels in HBeAg-positive patients were higher than those in HBeAg-negative patients. HBeAg-positive patients were older than HBeAg-negative patients. The alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in HBeAg-positive patients were higher than those in HBeAg-negative patients. The characteristics of the CHB patients are all shown in Table 3.

Prevalence of genotypic resistance mutations in CHB patients

The genotypic resistance mutations were detected in 41.72% (58/139) of the patients receiving NAs therapy. The detection rates of resistance mutations were 42.67% (32/75) and 40.63% (26/64) in patients with genotype B and genotype C, respectively. Drug resistance mutations were detected at positions rt80, rt173, rt180, rt181, rt194, rt202, rt204, rt236, and rt250, but were not observed at positions rt169, rt184, and rt233. The rtM204I/V mutation (34.5%; 48/139) was the most frequently occurred, followed by rtL80I/V (15.8%; 22/139) and rt180M (12.9%; 18/139) mutations. The prevalence of mutations in the RT region is shown in Table 4.

Table 2. Mutations positions associated with drug resistance.

Position	Drug	References
rt80	LAM	[6]
rt169	ETV	[6]
rt173	LAM	[6]
rt180	LAM, ETV, LDT	[6]
rt181	LAM, ETV, LDT	[6,7]
rt184	ETV	[18]
rt194	ADV, TNF	[7]
rt202	ETV	[18]
rt204	LAM, ETV, LDT	[6,7]
rt233	ADV	[26]
rt236,	ADV	[7]
rt250	ETV	[18]

LAM: lamivudine; ETV: entecavir; LDT: telbivudine; ADV: adefovir

Table 3. Characteristics of chronic hepatitis B patients

Characteristics	Value (n=139)
Average age, years (range)	37.6 (18-72)
Male/female gender, n (% males)	102/37 (73.38)
Mean HBV DNA, log_{10}copies/mL (± SD)	6.45 (1.69)
Positive/negative HBeAg status, n (% positive)	66/73 (47.48)
Genotype B/C, n (% genotype B)	75/64 (53.96)
Mean ALT level, IU/L(±SD)	107.21 (169.46)
Mean AST level, IU/L(± SD)	76.43 (137.76)

HBeAg: hepatitis B e antigen; ALT: alanine transaminase; AST: aspartate aminotransferase

Table 4. Incidence of drug-resistance mutations in the hepatitis B virus reverse transcriptase gene region.

Mutation	Associated drug	Number	Percentage (%)
No mutation	None	81	58.27
rtL80V	LAM/LDT	3	2.15
rtL80I	LAM/LDT	19	13.67
rtV173L	LAM	3	2.15
rtL180M	LAM/ETV	18	12.94
rtL180I	LAM/ETV	1	0.72
rtA181V	LAM/LDT/ADV	3	2.15
rtA181G	LAM/LDT/ADV	1	0.72
rtA194G	ETV	2	1.44
rtS202N	ETV	2	1.44
rtM204I	LAM/LDT/ETV	31	22.30
rtM204V	LAM/LDT/ETV	17	12.23
rtN236T	ADV	2	1.44
rtM250L	ETV	5	3.59
rtM250V	ETV	1	0.72

LAM: lamivudine; LDT: telbivudine; ETV: entecavir; ADV: adefovir

Table 5. Prevalence of resistance mutations in the reverse transcriptase gene in different treatment regimens.

Regimen	Cases, n	Duration of therapy, months (mean ± SD)	Mutation position	Number	Percentage
LAM	73	17.87 ± 6.95	rtM204V/I	42	57.53
			rtL80V/I	22	30.14
			rtL180M	19	26.02
			rtM250L	5	6.84
			rtV173L	3	4.11
			rtA181V	1	1.37
ADV	28	20.21 ± 6.05	rtA181V	2	7.14
			rtN236T	2	7.14
ETV	21	13.52 ± 7.26	rtA194G	2	9.52
			rtS202N	2	9.52
LDT	7	13.85 ± 4.29	rtM204I	2	28.57
LAM + ADV	6	19.33 ± 8.04	rtM204V/I	3	50.00
			rtA181G	1	16.67
			rtL180I	1	16.67
			rtA194T	1	16.67
LAM switched to ADV	4	27.25 ± 7.46[a] 20.25 ± 6.99[b]	rtL180M rtM204V/I	2 2	50.00 50.00
			rtM250V	1	25.00

[a] Duration of therapy with LAM alone. [b] Duration of therapy after switching to ADV; LAM: lamivudine; ADV: adefovir; ETV: entecavir; LDT: telbivudine.

The patients were treated with six different regimens, including monotherapy (LAM, ADV, ETV, LDT), combination therapy (LAM plus ADV), and sequential therapy (LAM switch to ADV). The prevalence of resistance mutations in various treatment regimens is shown in Table 5. Among patients who received LAM/LdT-based treatment, rtM204I/V mutations were harbored in 57.53%, 28.57%, 50.0%, and 50.0% of patients receiving LAM or LdT monotherapy, ADV add-on, or ADV switch-to therapy, respectively. Among patients who had received previous ADV treatment, the rtN236Tand rtA181V mutations were observed in 7.14% and 7.17% of cases, respectively.

Combination mutation patterns of the RT region in CHB patients

Combination mutations were found in 74.13% (43/58) of patients, and the incidence of combination mutations are shown in Table 6. Of the patients who received LAM/LDT-based treatment, the mutation rtM204I/V+ rtL180M ± rtL80I/V was most frequently observed (48.8%; 21/43), followed by rtM204I + rtL80I (34.88%; 15/43). Among patients who harbored rtM204 mutations, rtM204I often occurred with rtL80I (rtM204I + rtL80I [17/22; 42.5%] versus rtM204V + rtL80I [3/18; 7.5%], $p < 0.01$). However, rtM204V was often accompanied by rtL180M (rtM204V + rtL180M [18/18; 100%] versus rtM204I + rtL180M [4/22; 18%]; $p < 0.01$).

Additionally, rtA181V + rtN236T mutation was found in one patient with ADV treatment. Notably, rtA194G + rtS202N mutation was observed in two patients undergoing ETV treatment.

Discussion

The present study was a hospital-based survey of genotypic resistance profiles in 12 positions within the HBV RT region among 139 CHB patients who had received six different NAs treatment regimens in Huzhou, eastern China. The prevalence of genotypic resistance mutations was 41.72% (58/139), which is different from the prevalence reported in other countries [20-23], and is also different from studies in

Table 6. Combination mutation patterns in the reverse transcriptase gene of chronic hepatitis B patients.

Combination mutation patterns	Cases	Percentage
rtL80I + M204I	15	34.88
rtL180M + M204V	9	20.94
rtM204I + M250L	3	6.98
rtL80V + L180M +M204V	3	6.98
rtV173L + L180M +M204V	3	6.98
rtL80I + L180M +M204I	2	4.65
rtL180M + M204I	2	4.65
rtA194G + S202N	2	4.65
rtA181V + N236T	1	2.32
rtL180M + M204V + M250L	1	2.32
rtL180M + M204V + M250V	1	2.32
rtL180M + A181G + A194T + M204V	1	2.32
Total	**43**	**100.00**

other regions of China [14,17,24]. The discrepancy might be due to race and genotype of HBV in different countries, because the predominant HBV genotypes in Europe are A and D. However, the dominating HBV genotype in Korea is C. In China, though genotypes B and C are major genotypes, reports of the distribution of the genotypes in different region are inconsistent [19], which may also affect the frequency of genotypic resistance mutations. Additionally, the results might be influenced by not only sample size but also the duration and regimen of treatment.

In the present study, three classic primary mutations (rtM204V/I, rtA181V, and rtN236T) and other secondary mutations (rtL80I/V, rtV173L, rtL180M, and rt250L) were all detected. These finding were supported by previous studies [24,25]. The mutations at rt169 and rt184 residues responsible for ETV resistance were not detected. These results may due to the short treatment periods (no more than three years) and limited number of ETV-treated patients in this study.

In agreement with previous studies [14,17], the incidence of the rtM204V/I mutation was highest (34.53%; 48/139) in CHB patients who had received NAs; this mutation was associated with resistance to LAM, LdT, and ETV. RtM204V/I was the major mutation type (48/58; 87.1%) in the patients who were found to harbor drug resistance mutations. These findings were consistent with previous studies [24]. The rtM204V/I mutation is a very important and common mutation type in patients receiving NA treatment in eastern China. The reason for this phenomenon may be that LAM was the earliest drug with a low genetic barrier to resistance and had the longest application of anti-HBV therapy in China. Furthermore, rtM204V/I mutations are cross-resistant to other drugs (LdT and ETV) and may affect the efficacy of treatment [7]. Taken together, the risk of the propagation of rtM204 mutation strain might be a public health problem in the future because this resistant viral strain is difficult to clear completely [26].

Furthermore, our results showed that the incidence rate of rtM204I (64.58%; 31/48) was higher than that of rtM204V (35.42%; 17/48). The incidence rate of rtL80I mutation (86.36%; 19/22) was also higher than that of rtL80V (13.67%; 3/22). These results were congruent with those of previous reports [27,28]. These data suggest that some of the amino acid positions in the HBV RT region of NAs-treated patients prefer certain mutational patterns. Therefore, identification of the resistance mutation patterns, particularly AA residues, might be a benefit to the option of anti-viral therapy strategy.

In the present study, the rtL80I occurred in most of the patients with rtM204I, and the rtL180M often coexisted with the rtM204V, which was consistent with previous reports [14,24]. The phenotype analysis showed that rtL80I/V and rtL180M were compensatory mutations, which could recover the reduced replication efficiency caused by rtM204I/V mutant HBV strain [26,29].

In a previous study, other compensatory mutations, rtV173L or rtT184A (or S), were found clustering with rtM204V mutation in genotype D-infected patients [26]. However, only rtV173L mutation accompanied by rtM204V mutation was observed in genotype B and C patients [14]. In this study, rtV173L clustering with rtM204V mutation was found in only three LAM-treatment patients with genotype C HBV infection. These above data suggest that the HBV genotype might affect the evolution of the resistance development under selective pressure of LAM. However, more data must be collected to corroborate this presumption.

The rtI233V mutation, which had been reported to be correlated with ADV resistance, was not found in this study. Several studies had contradictory results on whether rtI233V substitution conferred ADV resistance of HBV [30-33]. Recently, a large-scale sample survey indicated that the incidence of rtI233V was low (0.71%) and that this mutation may partly serve as a compensatory mutation associated with ADV resistance [34]. We therefore believe that though rtI233V is not a common mutation in patients with ADV treatment, this genotypic resistance mutation is worth monitoring. Although rtM250I/L/V was defined as a mutation related to ETV resistance [35], in this study, the changes of AA at rtM250 were found in only six ETV-naïve patients. Among these patients, five had received LAM monotherapy, rtM204I + rtM250L mutations were observed in three cases, rtL180M + rtM204V + rtM250L mutations were found in one case, rtM250L mutation was found in another case. These results were in accordance with previous reports, which indicated that rtM250L might be selected in ongoing LAM monotherapy [36]. The mutation pattern of rtL180M + rtM204V + rtM250V was detected in one patient receiving LAM who had switched to ADV sequential treatment. Whether the rtM250V mutation arose during the LAM or ADV treatment was difficult to judge. These data implied that rtM250 mutation might be also select by other NAs besides ETV. The efficacy of ETV in rescue therapy for these ETV-naïve patents harboring the rtM250L/V mutation requires follow-up study.

Interestingly, rtA194G + rtS202N combination mutations were found in two viral breakthrough patients receiving ETV monotherapy, but biochemical breakthrough did not occur. It has been reported that rtA194T was associated with ADV and TDF resistance [7,37] and rtS202C/G/I was associated with ETV resistance [35.36]. To our knowledge, this mutation type has not been reported before. The clinical and biological significance of the rtA194G + rtS202N combination mutation needs to be further investigated.

Conclusions

The results of this study show complex profiles of resistance mutations in patients receiving NAs in clinical practice in eastern China. RtM204V/I are the current major mutation of resistance to drugs in CHB patient treatment with NAs. Several mutations have the potential to contribute to the development of cross-resistance to rescue therapy with other NAs in the future. These data suggest that monitoring HBV genotypic resistance could guide and optimize the antiviral strategies and reduce the occurrence of clinical drug resistance.

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