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Original Research

Relationship between hepatitis B virus reverse transcriptase 181 mutation and S gene mutation in hepatitis B virus chronically infected patients

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Abstract: This study aims to explore clinical significance of HBV rt181 mutation. Serum samples were collected from 226 CHB patients with no anti-viral treatment, and 72 patients with adefovir dipivoxil treatment over 1 year. HBV genes of reverse transcriptase regions were amplified by nested PCR. HBV DNA in pre-S/S regions sequences were determined by sequencing. Mutations in HBV were characterized by mutational analysis. Results indicated that resistant mutation was found in 16 samples (7.08%) with no anti-viral treatment. It showed higher prevalence in patients with adefovir dipivoxil treated samples 30/72(41.67%). Mutation sites of pre-existing and adefovir dipivoxil induced resistance were different (adefovir dipivoxil induced resistance mode is rtA181T/V, and pre-existing mode is the other). Resistance mutation was found just in genotype C patients. Among 25 containing rtA181T/V mutation patients, 7 rtA181T mutation cases with sW172L, 6 rtA181T mutation cases with sW172*, 12 rtA181Vmutation cases with sL173F. In conclusion, mutation sites of pre-existing and adefovir dipivoxil induced resistance were different. HBV genotype C is prone to occur resistance mutation than genotype B. rtA181T mutation was accompanied with not only sW172 * mutation, but also sW172L mutation, rtA181V mutation was accompanied with sL173F mutation or Pre-S2 initiation codon to termination mutation.

Key words: Hepatitis B virus, genotype, rt181, resistant mutation, reverse transcriptase.

Introduction

Hepatitis B virus (HBV) infection leads to chronic hepatitis B (CHB), cirrhosis and even hepatic carcinoma. There are no effective methods to eliminate hepatitis B virus. 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 (1,2). Viral resistance will occur following the longer time of NAS treatment. The 181 amino acid mutation in HBV reverse transcriptase region challenges NAS, for the induction of resistance to adefovir dipivoxil and the less sensitivity to lamivudine, entecavir, telbivudine and tenofovir (3). It is a cross-resistance locus of NAS drugs (4). This mutation was not only induced in adefovir dipivoxil treated patients, but also in telbivudine treated patients (5,6). The resistance mutation existing in no antiviral treated patients is called pre-existing resistance, the previously study reported that rtA181V/T mutation was also existed in no antiviral CHB patients (7). *In vitro* study results indicated that the rtA181T/V mutational virus was resistant to most NAS agents. Lai (8) reported a case that HBeAg positive but HBsAg negative which HBsAg negative was due to the mutation of rtAl8IWsWl72*. Their *in vitro* results demonstrated that rtAl81T/sWl72* mutation could induce SV40 and the oncogene c-Myc, thus enhancing cancer progression. rtAl81T/sWl72* mutation could promote tumorigenesis in nude mice. Recently some clinical observation revealed that rtA181T/sW172* mutation in lamivudine-resistant patients promoted HCC progression (9).

The emergence and takeover of HBV variants carrying mutation(s) in the preS/S genomic region is a fairly frequent event that may occur spontaneously or may be the consequence of immunoprophylaxis or the use of antiviral drugs. A growing number of studies are pointing out the considerable importance of preS/S variants including those able to escape the vaccine-induced anti-HBV neutralizing antibodies as well as those frequently associated with severe forms of acute and chronic liver disease and HCC development (10-12).

In clinical, we observed that rt181 mutation existed not only in HCC patients, but also in CHB patients. In this study we will investigate the difference between pre-existing resistance and adefovir dipivoxil induced resistance. Our study showed that 2 Pre-S2 patients were the initiation codon mutation and both were rtA181V mutation, 6 cases were rtA181T / sW172 * mutation. These 8 patients were all genotype C patients. The Pre-S region mutations and C genotype are the risk factors of HCC, and our observation shows they were all CHB patients. if such a mutation will really increase immune evasion and promote the progression of liver disease, it's necessary for such patients to be long-term follow-up and close observation to detect changes and intervene as soon as possible. Of course it is necesseory to explore the clinical significance of HBV rt181 mutation.

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Table 1. HBV genotyping reference sequence.

Genotype	GenBank HBV Gene sequence encoding
A	AF090842, X02763, X51970
B	AF100309, D00329, AB033554
C	AB014381, M12906, X04615
D	X65259, M32138, X85254
E	X75657, AB032431
F	AB036910, AF223965, X69798
G	AF160501, AF405706, AB064310
H	AY090454, AY090457, AY090460

Materials and Methods

Patients and serum samples

HBsAg positive and HBV DNA positive serum samples were collected from the affiliated hospital of Xuzhou medical college from June 2013 to December 2014. There are 236 male and 62 females whose age range from 15 to 68 years old, with the mean age was 35.16 years. There are 226 CHB patients with no antiviral treatment, and 72 patients with adefovir dipivoxil treatment for over 1 year but the HBVDNA were still positive and got virologic breakthrough. Diagnose of the patients were according to the guideline of prevention and treatment for chronic hepatitis B (2010) (2). 2ml peripheral venous blood were collected from each patient and centrifuged for 20 min at 2000 rpm. Serum was stored at -80°C. Informed consent was obtained from each patient and the study was approved by Institute Research Ethics Committee.

Nested polymerase chain reaction and direct sequencing

HBV DNA was extracted from serum samples using the commercial kit (Shanghai Shenyou Biotech Company, China). HBV genes of the P regions were amplified by nested PCR. PCR primers were supplied in the kit. PCR reaction was carried out in 30 mL containing 20 µl buffer, 2 µl $MgCl_2$, 2 µl primer, 2 µl Taq DNA polymerase and 2ul template. The amplicons were isolated by agarose elelectrophoresis and purified by ethanol precipitation. Both strands of PCR products were directly sequenced in the forward and reverse directions using an ABI 3130 sequencer and commercial kit BIGDYE (ABI).

HBV genes of the pre-S/S regions were amplified by PCR. PCR primers were 5'-GGGTCACCATATTCTTGGGAAC-3' (nt2814-2835) and 5'-AGCCCAAAAG ACCCACAATTC-3' (nt 1015-995) (13) . PCR reaction was carried out in 50 µL containing 25 µL buffer, 4 µL 2.5 mmol/L deoxynucleosidetriphosphates (dNTP), 2 µL 10 mmol/L sense and antisense primers, 1.5 U Platinum Taq DNA polymerase (Invitrogen, shanghai, China) and 4 µl template. The amplicons were isolated by agarose elelectrophoresis and purified by ethanol precipitation. Both strands of PCR products were directly sequenced at Nanjing Springen Company. The data were analyzed and HBV genotypes were assigned using NCBI Viral Genotyping Tool (www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi), Genotype reference sequences were shown in table 1. Multiple comparisons were made by the software BioEdit.

Statistical analysis

Data were expressed as means and standard deviations (SDs). Differences were analyzed using the Student's t-test for continuous variables and the Chi-square (χ^2) test for categorical variables. A P value less than 0.05 was considered statistically significant.

Results

Comparison of the age, sex and the levels of HBV DNA between genotype B and genotype C patients

As shown in Table 2, 2 genotypes were identified in 298 CHB patients, including 281 (94.3%) C genotype and 17 (5.7%) B genotype. while the HBV genotypes A, D, E, F, G, and H were not detected. The characters of each patient were collected and analysed. The average age of genotype B patients was 30.18±9.15years and the average age of genotype C patients was 35.46±11.48 years. There was no significant difference between the age of these two genotype patients (P=0.271). In both two genotype patients, there was more male than female, but no significant difference was found (P=0.274). Levels of HBV DNA were also comparable in these two genotypes (P=0.71).

As shown in Table 3, 72 patients had got adefovir dipivoxil treatment before and 226 were not among the 298 patients. In the adefovir dipivoxil-treated group, 30 patients got drug resistance mutation and 42 were not, the mutation rate was 41.67 %. In the no-adefovir dipivoxil-treated group, 16 patients got drug resistance and 210 were not, the mutation rate was 7.08 %. It is shown

Table 2. Characteristics of hepatitis B virus genotype B and C.

Genotype	B (n=17)	C (n=281)	*P*
Age (years; mean±SD)	30.18±9.15	35.46±11.48	0.271
Gender (M/F)	15/2	221/60	0.274
HBV DNA (log_{10}IU/ml)	6.74±1.32	6.47±1.28	0.71

Comparison of the age, sex and the levels of HBV DNA between genotype B and genotype C patients, there is no statistical significance (P>0.05).

Table 2. The mutation rate between the Adefovir dipivoxil treated and untreated patients.

Group	Mutation case	wild case	total	Mutation rate (%)
Treated	30	42	72	41.67
Untreated	16	210	226	7.08

It is shown that Adefovir dipivoxil treatment could significantly induce the drug resistance (p=0.000).

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Sequencing of no antiviral CHB patients

16 (7.08 %) patients were identified with drug resistance mutation among 226 no antiviral CHB patients. 6 patients were associated with lamivudine resistance, whose mutation types were 1 rtV207L and 5 rtS213T. 8 patients were associated with adefovir dipivoxil resistance, whose mutation types were 1 rtA181T, 3 rtV214A and 4 238D/S/T. 2 patients were associated with both, whose mutation types were 1 rtV207I+ rtN/H 238D and 1 rtS213T + rtN/H 238S.

Sequencing of Adefovir dipivoxil-treated CHB patients

30 (41.67 %) patients were identified with drug resistance mutation among 72 adefovir dipivoxil-treated CHB patients. 1 patient was associated with lamivudine resistance, whose mutation type was rtS213T. 25 patients were associated with adefovir dipivoxil resistance mutation, whose mutation types were 7 rtA181T, 10 rtA181V, 2 rtA181T +rtN236T, 1 rtA181T+rt238T, 2 rtA181V+rt238S, 1 rtV214A and 2 rtN236T. 4 patients were associated with both, whose mutation types were rtA181T +rtV207I, rtA181T +rtV207I +rtN236T, rtS213T+ rtV214A, rtN236T+ rtM250L. Definite data was shown in Table 4.

Correlation between rtA181T/V and S gene mutation

In the 5 rtA181T mutational patients and 2 rtA181T +rtN236T mutational patients, their 172 amino acid of S gene were mutated from Trp to Leu, which was named rtA181T/sW172L (Figure 1A). The sw182 amino acid of S gene from 1 rtA181T mutational patient was mutated to termination codon TGA. The 172 amino acid of S gene from 3 rtA181T mutation patients and 3 multiple mutation patients containing rtA181T (rtA181T +rtV207I, rtA181T +rtV207I+ rtN236T and rtA181T+rt238T) was mutated to termination codon TGA, which was named rtA181T/sW172* (Figure 1B, C, D). The 173 amino acid of S gene from 10 rtA181V mutation patients and 2 rtA181V+rt238S mutation patients were mutated from Leu to Phe, which was named rtA181V/sL173F. Definite data was shown in Table 5.

Table 5. The presence of specific mutations in S regions with HBV rtA181T/V mutation.

Mutation	case
rtA181T/sW172L	7
rtA181T/sW172*	6
rtA181V/sL173F	12
total	25

Table 6. Comparison of the age and serum virus burden in rt181 mutation patients.

Characteristics	rtA181T	rtA181V	P
Age (years)	45.41±10.93	42.00±6.72	0.427
HBV DNA (log_{10}IU/ml)	6.43±0.94	6.45±0.85	0.956

There was no significant difference between these two mutation patients.

Comparison of the age and serum virus burden in different mutation patients

The average age of rtA181T patients was 45.41±10.93 years, the average age of the rtA181V patients was 42.00±6.72 years. There was no significant difference between these two mutation patients (p=0.427). HBV DNA levels of rtA181T patients were 6.43±0.94, and HBV DNA levels of rtA181V patients were 6.45±0.85. There was no significant difference between these two mutation patients (p=0.956). Definite data was shown in Table 6.

Discussion

rtA181T/V mutation can be either natural mutation or be induced by the NAS antiviral drugs. Recently rtA181T/V mutation was detected in the lamivudine, adefovir dipivoxil and telbivudine treated patients (13-16). rtA181T/V is a cross-resistance locos for NAS, and resistant to almost every NAS drugs (4), this challenges the later treatment a lot. At present, there is no unified

Table 4. Distributation of HBV mutations between the Adefovir dipivoxil treated and untreated patients.

Mutation	Untreated group	Treated group	Total
rtA181T	1	7	8
rtA181V	0	10	10
rtS213T	5	1	6
rtV214A	3	1	4
rtV 207L	1	0	1
rtN 236T	0	2	2
rtN/H 238D/S/T	4	0	4
rtA181T +rtV207I	0	1	1
rtA181T +rtV207I+ rtN236T	0	1	1
rtA181T +rtN236T	0	2	2
rtA181T+rt238T	0	1	1
rtA181V+rt238S	0	2	2
rtV207I+ rtN/H 238D	1	0	1
rtS213T+ rtV 214	0	1	1
rtS213T + rtN/H 238S	1	0	1
rtN 236T+ rtM 250L	0	1	1
Wild type	210	42	252
total	226	72	298

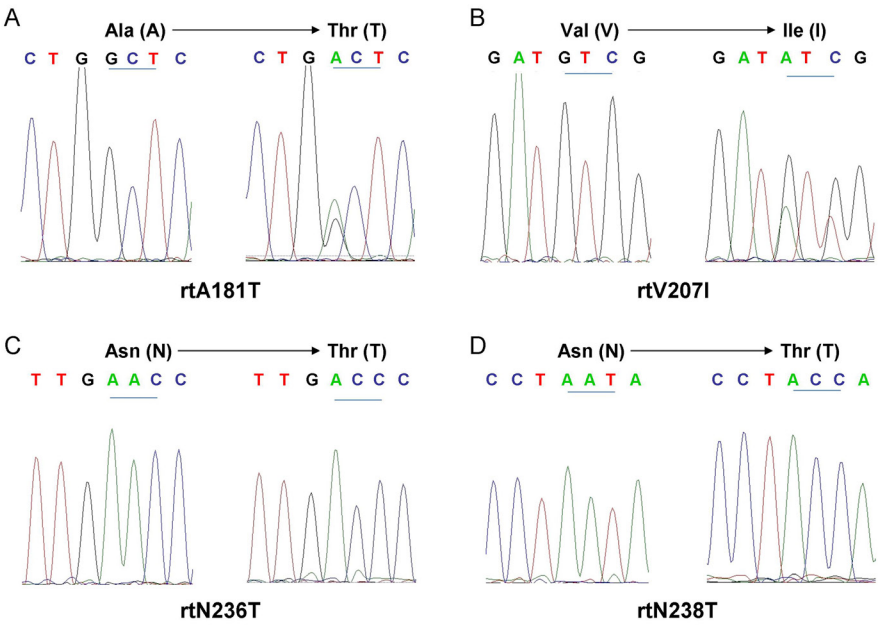

Figure 1. The genotyping maps for the mutations of rtA181T (A), rtV207I (B), rtN236T (C) and rtN238T (D).

recommendation and evaluation standard of the later treatment of rtA181T/V patients. EASL (17) suggested the combination of emtricitabine with entecavir or tenofovir, but the inhibition of rebound mutational virus needs further observation and follow-up.

In our study we found that 226 CHB patients without use any of antiviral drugs, 16 pre-existing genotype resistance mutations were detected and all belong to genotype C. 6 patients were associated with lamivudine resistance. Their mutation types were 1 rtV207L and 5 rtN/H 238D/S/T. 8 patients were associated with adefovir dipivoxil resistance. Their mutation types were 1 rtA181T, 3 rtV214A and 4 rtN/H 238D/S/T. 2 patients were associated with both. Their mutation types were rtV207I+ rtN/H 238D and rtS213T + rtN/H 238S. No drug resistance was detected in genotype B patients. Pre-existing resistance to lamivudine and adefovir dipivoxil existed in the CHB patients. The pre-existing resistance mutation rate was significantly higher in the genotype C patients comparing with genotype B patients. By the way, there were more genotype C patients in the northern China and these NAS drug should be avoid to those CHB patients with rtA181T/V mutation.

In the 72 adefovir dipivoxil treated CHB patients, 70 patients were genotype C patients and 2 patients were genotype B patients. 30 (41.67%) resistance mutation were detected. 1 patient was associated with lamivudine resistance. His mutation type was rtS213T. 25 patients were associated with adefovir dipivoxil resistance. Their mutation types were 7 rtA181T, 10 rtA181V, 2 rtA181T +rtN236T, 1 rtA181T+rt238T, 2 rtA181V+rt238S, 1 rtV214A and 2 rtN236T. 4 patients were associated with both drugs. Their mutation types were rtA181T +rtV207I, rtA181T +rtV207I +rtN236T, rtS213T+ rtV214A, rtN236T+ rtM250L. There was only one patient with rtN 236T mutation was genotype B among the above mentioned mutational cases, the others were all genotype C cases. From these results we can see that the gene mutation induced by adefovir dipivoxil was different from pre-existing resistance mutation. Adefovir dipivoxil induced mutation was mainly rtA181T/V mutation and pre-existing resistance mutation occurred mainly in other sites. Both drug resistance occurred in genotype C patients. Moreover, the results indicated that the patients treated with adefovir dipivoxil (1/72) have lower lamivudine resistance mutations compared to the patient treated without adefovir dipivoxil (2/226), but without significant differences. This result suggests that the adefovir dipivoxil may inhibit the mutations of the CHB through the some specific pathway, which would be explored in the following study.

Furthermore, the clinical data has not been illustrated the unique features other than the age and the gender. Our previous observation showed that the drug resistant mutation is not related to the duration of adefovir dipivoxil tratment.

As the RT gene and the S gene are completely overlapping, the resistance mutation of HBV RT gene may result in the mutation of S gene and further more affect the HBsAg coding and antigen activity, thus may produce immune escape variants (18-21). The rtA181T/sW172* strain generates a termination codon in advance and produces an interceptive S protein. Recently *in vitro* and *in vivo* study results suggest the potential carcinogenicity of rtA181T/sW172* strain (22-25). Their study results revealed that the rtA181T/sW172* stain secreted virus particles abnormally, resulting in the accumulation of interceptive S protein and a decreased secretion of WT strain, leading to a lower extracellular viral burden. If judging the virological breakthrough only by the serum levels of viral, relevant resistance mutations will not be found (22). Wang et al. (26) reported the correlation between Pre-S gene mutation and incidence of hepatocellular carcinoma by META analysis. Their study contains 2837 HBV infected patients and 1246 patients with HCC. Their results show that the Pre-S gene mutation incidence was higher in the HBV infected patients with HCC comparing with the patients without HCC. The clinical study in limited cases found that this mutation was correlated with a higher HCC incidence. Otherwise, rtA181T/ sW172* mutation in CHB was found in our study. And in those cases there were no HCC signs. In order to further confirm its carcinogenicity, rtA181T/sW172* patients need further observation and follow-

up.

2 Pre-S2 initiation codon occurred termination codon mutation and 6 sW172* mutation were detected in our study. The S region of HBV consists of Pre-S1 gene (nt2854~3210), Pre-S2 gene (nt3211~154) and S gene (nt155~832). Pre-S1, Pre-S2, S are adjacent genes, each with a independent promoter, but share the same terminator. S gene and Pre-S gene are areas which were prone to mutate, so the expression of the antigenic protein may change pluralisticly. Recent study revealed that the mutation of HBV Pre-S gene may affect the packaging, secretion, infection and immunogenic of the HBV virus and was correlated with HCC (27). Pre-S gene mutation rate increased with the progression of HCC, Pre-S mutation rate of HCC patients was significantly higher than that of other patient (28). Utama et al. (29) reported that Pre-S2 initiation codon mutation was an independent factor affecting progression of liver disease. The mutation was correlated with advanced liver disease. Pre-S2 mutations in HBV genotype C correlated with liver disease closely. Meanwhile, Pre-S2 protein deficiencies can cause T cells and B cells epitopes lost, thus they can not produce enough neutralizing antibodies to remove viruses. HBV could survival in the host body, causing chronic HBV infection, leading to exacerbation and viral immune escape. It can also affect the host immune response and result in immune failure (30). Li et al. (31) found that Pre-S gene deletion mutation and Pre-S2 gene initiation codon mutation is an independent risk factor for liver cancer, and the Pre-S gene deletion mutation is not found early in the disease, but occurred in the process of hepatocellular carcinoma gradually. Our study showed that 2 Pre-S2 patients were the initiation codon mutation and both were rtA181V mutation, 6 cases were rtA181T / sW172 * mutation. These 8 patients were all genotype C patients. The Pre-S region mutations and C genotype are the risk factors of HCC, and in our observation they were all CHB patients. If such a mutation will really increase immune evasion and promote the progression of liver disease, it is necessary for such patients to be long-term follow-up and close observation to detect changes and intervene as soon as possible.

Taken together, our study indicated that the pre-existing resistance exists in the CHB patients. The mutation sites of pre-existing antiviral resistance and adefovir dipivoxil induced drug resistance were different (adefovir dipivoxil induced drug resistance mode is rtA181T/V, and pre-existing antiviral resistance mode is on other sites). But the resistance mutation occurs in genotype C patients. During the treatment of adefovir dipivoxil, the incidence of rtA181T/V mutation is higher under the condition of virological rebound or poor treatment. In this experiment rtA181T mutation was accompanied with sW172L or sW172 * mutation. rtA181V mutation was accompanied with sL173F mutation or Pre-S2 initiation codon to termination mutation. It is unclear whether these mutations would increase the difficulty of treatment or the risk of liver cancer. The mechanism of its carcinogenic and its clinical significance needs further study.

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