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Original Research Article

Preliminary study on the relationship between recurrence and quasispecies characteristics in P region of hepatitis B virus genome of chronic hepatitis B patients treated with lamivudine

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Abstract

Purpose: *To investigate the characteristics of quasispecies in the P region of hepatitis B viral (HBV) DNA of chronic hepatitis B (CHB) patients treated with lamivudine (LAM), and its effect on HBV relapse after drug withdrawal in CHB patients who met drug cessation criteria.*

Methods: *A total of 43 patients with chronic HBV infection, who had undergone LAM therapy, were enrolled in this study. Treatment was discontinued for patients who met therapeutic criteria set by relevant Asian-Pacific regions. Polymerase chain reaction (PCR) was used to amplify the genome in P region of serum rcDNA before treatment, cccDNA during drug cessation period, and serum rcDNA at relapse. Quasispecies cloning and sequencing were performed to identify variable sites in HBV P region.*

Results: *Mutations in P region of baseline serum rcDNA were detected in 30 CHB patients, with N/H238T (14/30), L/F/Q/R267H (12/30), V278T (12/30), D134E/I (11/30), and T222A (9/30) having highest rates. In hepatocellular cccDNA P region during drug withdrawal, most detectable mutations were L/F/Q/R267H (25/43), V278T (18/43), N/H238T (15/43), D134E/I (14/43), and T222A (11/43). During relapse, the highest detectable mutation rates in serum rcDNA P region were N/H238T (12/19), L/F/Q/R267H (10/19), T222A (10/19), and V278T (8/19).*

Conclusion: *High mutation rates of T222A and N/H238T in P region of HBV DNA increase the risk of relapse in patients. As a result, patients are susceptible to relapse after drug withdrawal.*

Keywords: *Hepatitis B Virus, Hepatocellular cccDNA, Mutation*

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INTRODUCTION

Low fidelity of reverse transcriptase (RT) during Hepatitis B Virus (HBV) replication leads to abnormal RNA splicing and defects. As a result, a mixture of strains known as quasispecies, which are highly related genetically, exists in the host, rather than as a single strain with a unique

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sequence. The composition and evolution of quasispecies may influence clinical manifestations and prognosis of HBV infections [1]. Currently, studies on HBV quasispecies mainly focus on drug response, drug resistance, and disease progression. However, there is limited research on whether complexity and changes of quasispecies influence HBV relapse after drug withdrawal and its related mechanism. This study aims to investigate quasispecies features of P region in HBV DNA among chronic HBV (CHB) patients receiving LAM therapy using cloning techniques [2]. The study also aims to determine the effects of these quasispecies on HBV relapse after drug withdrawal.

METHODS

Research scheme

Inclusive criteria

A total of 43 patients with chronic HBV infection who had been subjected to LAM therapy (25 of them in the stage of initial treatment while 18 retreated) were enrolled. Treatment of patients was terminated after they met the criteria as laid down by *Guideline on Prevention and Management of Chronic Hepatitis B in Asian-Pacific regions*, or after the period of treatment had been extended. Then all enrolled patients accepted liver puncture.

Patients in lamivudine (LAM) preliminary treatment group had never been subjected to HBV anti-virus therapy. They were given LAM treatment in the course of primary therapy, and none of them underwent virological breakthroughs during treatment.

Patients in LAM retreatment group who had LAM-resistant HBV infection underwent retreatment with nucleotide analogs (Nas). After Nas retreatment, patients who did not meet the criteria for LAM cessation (with a mean time of drug discontinuation exceeding six months) discontinued drug administration. All patients included in the two groups were required to meet the following criteria: a diagnosis of CHB infection according to guidelines, provision of informed consent, a liver puncture for patients meeting drug withdrawal criteria as specified in guidelines, and willingness to participate in follow-up studies (a follow-up period of more than 6 months for non-recurrent patients, and good compliance with timely return visits). This study was approved by the Ethics Committee of Minzu Hospital of Guangxi Zhuang Autonomous Region (approval no. 20-EC-11), and complied with international guidelines for human studies. Signed written informed consent was obtained from patients and/or guardians.

Exclusion criteria

Patients with HBV infection who were co-infected with viral hepatitis C (HCV), viral hepatitis D (HDV), acute hepatitis, AIDS, liver cirrhosis, and liver cancer were excluded from the study. Additionally, patients who had received immunosuppressive agents and interferon therapy were also excluded.

Retrospective study and indices determined

A retrospective survey was conducted on clinical data of patients who met LAM drug cessation criteria according to the Guideline. Hepatitis B viral DNA levels in serum was determined using the fluorescence quantitative PCR (Shanghai Kehua Bioengineering Co. Ltd, Shanghai, China) method and determination limit was 1.0×10^3 cps/mL. The level of alanine transaminase (ALT) was tested using velocity method (Shanghai Kehua Bioengineering Co. Ltd, Shanghai, China). Serologic HBV markers were measured using time-resolved fluoroimmunoassay (TRFIA) (Shanghai Xinbo Bioengineering Co. Ltd, Shanghai, China) method.

All tests were carried out by professionals at the Testing Center of the First Affiliated Hospital of Guangxi Medical University. Follow-up continued after drug withdrawal, with the same indices to be detected every month within the first 6 months and every 2 months afterward until HBV-DNA recurrence (two successive detections of HBV DNA > 1.0×10^3 cps/mL). For non-recurrent patients, the follow-up period was more than 6 months (the date of last follow-up served as the end-point of study).

Sampling and storage

Baseline serum samples in the course of treatment (10 mL), liver tissues after drug withdrawal criteria were met (≥ 5 mg), and serum after HBV relapse (10 mL) were collected. All serum samples were stored in refrigerator (-40 °C) for later use. Liver tissues (obtained from liver puncture) were kept in refrigerator (-80 °C).

DNA cloning and sequencing

Extraction of HBV DNA

Hepatitis B viral DNA (HBV DNA) in 200 – 1000 mL serum (according to HBV DNA loads) and in liver tissues (≥ 5 mg) were extracted using DNA extraction kits (Qiagen, Germany) according to specified procedures. The full-length genome of HBV cccDNA was acquired through RCA method and SPEI endonuclease digestion.

Amplification and purification of P region of HBV

Searching for complete sequences of HBV gene (Gen bank NO.AY123041), and primers according to the conserved sequence of HBV genome were designed with primer-design software Premier 5.0. Fragments (537 bp) of primers were amplified including the codon which was coded by Nas-resistance sites, such as rt169, rt173, rt180, rt181, rt202, rt204, rt236 and rt250 (synthesized by Shanghai Kehua Bioengineering CO. LTD, Shanghai, China). Outer primers: upstream primer 5'-TGTACTTTCCTGCTGGTGGCTCCAGT-3' and downstream primer 5'-CGAGCAACGGGGTAAA GGGTC-3', Inner primers: upstream primer 5'-GTATGTTGCCCGTTTGTCCTC-3', and downstream primer 5'-CGTTGACAAACTTTCCA ATCA-3'. For PCR system, conditions used were: 5 min at 95 °C for denaturation; 35 amplification cycles (35 sec at 95 °C, 35 sec at 57 °C, 120 sec at 72 °C), and 10 min at 72 °C for elongation. Products of PCR-amplification were analyzed by electrophoresis on 1.5 % agarose gels and visualized on a UV transilluminator (Minibis gel imaging system, DNR, Israel), and then purified through gel isolation (QIA Quick Gel Extraction Kit, Qiagen, Germany).

Construction, identification and sequencing of clones

Products of HBV rcDNA and hepatocyte cccDNA P region (3 and 1 μL, respectively) were cloned into lpGEM-T vector, then transformed into 50 μL Escherichia coli cells (JM109) with a 60 min ice bath, and then a 60 sec heat shock for resistance recovery. After 4 min of ice bath, 250 μL of LB medium was added, and the sample was incubated under a constant temperature shaking method (37 °C for 1.5 h, at 300 rpm/min). After centrifugation of bacterial liquid sample, 200 μL of the supernatant was coated on an LB plate (100 μL/mL ampicillin, 7 μL ITPG, 20 μL X-gal), and incubated under the constant temperature (37 °C for 14 – 16 h). Clones were randomly selected and suspended in sterile water (forming a kind of bacterial liquid). Polymerase chain reaction (PCR) Identification was carried out for TERT encoding parts which were cloned from this bacterial liquid. The PCR primers of the TERT encoding parts were: M13F 5'-TGTAAAACGGCCAGT-3', and M13R:5'-CAGGAAACAGCTATGACG-3'. conditions for 10 μL PCR system were as follows: at 95 °C for 5 min; 30 amplification cycles (35 sec at 95 °C, 35 sec at 56 °C, 1 min at 72 °C); 72 °C for 10 min. Products of PCR amplification were analyzed through electrophoresis on 1.5 % agarose gels, and products with objective bands were treated as positive clones. A total of 14 – 25 positive clones of each sample were selected for amplifying, and sequenced by Huada Biological Company.

Statistical analysis

Data were analyzed with Statistical Package for Social Science (SPSS) software (version 26.0). Enumeration data were expressed in numbers and percentages (n (%)) and measurement data were reported in mean ± standard deviation. Student *t*-test and rank sum test was used to compare differences between means. Detectable rates of variable sites were compared using chi-square test, and life table method was used to calculate the cumulative relapse rate. $P < 0.05$ was considered statistically significant.

RESULTS

General information on patients

A total of 43 patients with chronic HBV infection who had received liver puncture at the Department of Infection of the First Affiliated Hospital of Guangxi Medical University (25 of them were in initial treatment stage and 18 were retreated) were enrolled. The re-treatment regimen of 18 patients was as follows: one was retreated with LAM, two with LDT, seven with ADV, two with ETV, and six accepted a combination therapy with LAM and ADV. The general information on the 43 patients is shown in Table 1.

Connections between recurrence and mutations in the HBV P gene

Relationship between recurrence and mutation in rcDNA P region in baseline serum

In P region of baseline serum rcDNA, highest detectable rates of mutation sites were: N/H238T (14/30), L/F/Q/R267H (12/30), V278T (12/30), D134E/I (11/30) and T222A (9/30), of which the highest in preliminary treatment group were D134E/I (9/16), L/F/Q/R267H (6/16), N/H238T (6/16), V278T (6/16) and T222A (6/16). The highest in retreatment group were N/H238T (8/14), L/F/Q/R267H (6/14), V278T (6/14) and T222A (3/14). Data in Table 2 showed that in P region of baseline rcDNA, the detectable rate of T222A in recurrent patients was significantly higher than that in non-recurrent patients ($p < 0.05$).

Relationship between relapse and mutation of P region in cccDNA of liver tissues in stage of drug withdrawal

In P region of hepatocellular cccDNA, highest detectable rates of mutation sites in drug withdrawal stage were L/F/Q/R267H (25/43), V278T (18/43), N/H238T (15/43), D134E/I (14/43) and T222A (11/43), among which the highest in preliminary treatment group were D134E/I (12/25), L/F/Q/R267H (10/25), V278T (8/25) and N/H238T (7/25), and the highest in retreatment group were L/F/Q/R267H (15/18), V278T (10/18) and N/H238T (9/18) (Table 3). In hepatocellular cccDNA P region, detection rate of N/H238T in recurrent patients was significantly higher than that in non-recurrence ones ($p < 0.05$), while L/F/Q/R267H and D134E/I rates were significantly higher in non-relapsing patients than those in relapsing patients ($p < 0.05$). In initial treatment group, detectable rates of L/F/Q/R267H and D134E/I were significantly higher in non-relapsing patients than in relapsing patients ($p < 0.05$). In retreatment group, detectable rate of N/H238T was significantly higher in recurrence patients than in non-recurrence patients ($p < 0.05$).

Mutations in the P region of serum rcDNA at the time of recurrence

At recurrence time highest detectable rates of mutation sites in P region of serum rcDNA were N/H238T (12/19), L/F/Q/R267H (10/19), T222A (10/19) and V278T (8/19), among which the highest in primary treatment group were N/H238T (6/10), L/F/Q/R267H (5/10) and T222A (5/10), and highest in retreatment group were N/H238T (6/9), V278T (6/9), L/F/Q/R267H (5/9) and T222A (5/9).

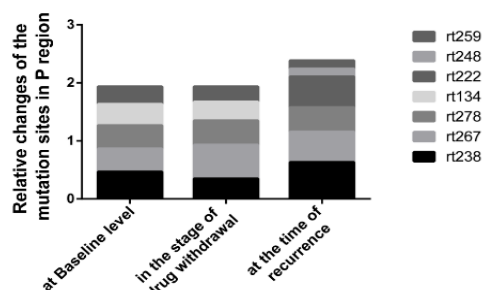

Figure 1: Changes in the highest rates of mutation sites in the P region at baseline level, in the stage of drug withdrawal and at the time of recurrence

DISCUSSION

Currently, anti-hepatitis B virus (anti-HBV) Nas drugs available in China are divided into 3 groups namely; left-lateral deoxycytidine analogues, including LMV, LDT; acyclic phosphate, e.g. ADV; and alkylcyclopentanes including deoxyguanosine analogues, e.g ETV. The reason Nas may lead to drug-resistance mutation of serum rcDNA is that during long-term anti-viral therapy adaptive drug-resistance mutations in HBV polymerase/reverse transcriptase gene may be induced by drug-selected pressure. In the course of rapid replication of HBV, reverse transcription region of DNA polymerase failed to recognize wrong nucleotides due to a lack of proofreading capacity. Thus, a relatively higher rate of gene mutation occurs during viral replication.

Table 1: Basic information on 43 patients with chronic HBV infection

Item	Initial treatment group	Re-treatment group	Total
Number (n)	25	18	43
Recurrence (n, %)	13 (52)	9(50)	22(51.2)
Age (years)	30.24±11.13	33.89±1183	31.77±1.43
Male (n, %)	14 (56)	15 (83.33)	29(67.44)
Baseline HbeAg(+) (n, %)	14 (56)	5 (27.78)	19(44.19)
Average period of treatment (months)	39.08 ± 21.66	35.72 ± 9.96	37.67±17.64
Average time of recurrence (months)	11.69±14.04	3.78±4.47	8.45±11.67
Genotypes (n, %)			
B	5(20)	2(11.11)	7 (16.28)
C	17(68)	14(77.78)	31 (72.09)
B+C	3(12)	2(11.11)	5(11.63)
Qualified samples (n, (%))			
Baseline serum	16(64)	14(77.78)	30 (69.77)
Standard liver tissues	25(100)	18(100)	43 (100)
Serum at recurrence	10(76.93)	9(100)	19 (86.36)
Baseline serum, serum at the time of recurrence and standard liver tissues obtained at the same time	7 (53.85)	8(88.89)	15(68.18)

Table 2: Recurrence rates and mutation sites in P region of baseline rcDNA

Initial treatment (N = 16)			Retreatment (N = 14)			Total (N = 30)		
Recurrence N=10 (n, %)	*Non-recurrence N=6 (n, %)*	*P-value*	*Recurrence N = 8 (n, %)*	*Non-recurrence N=6 (n, %)*	*P-value*	*Recurrence N=18 (n, %)*	*Non-recurrence N=12 (n, %)*	*P-value*
2(20)	4(66.67)	0.062	3(37.5)	3(50)	0.64	5(27.78)	7(58.33)	0.094
3(30)	3(50)	0.424	5(62.5)	3(50)	0.64	88(44.44)	6(50)	0.765
4(40)	5(83.33)	0.091	1(12.5)	1(16.67)	0.825	5(27.78)	6(50)	0.216
5(50)	1(16.67)	0.182	3(37.5)	0(0)	0.091	8(44.44)	1(8.33)	0.034
3(30)	3(50)	0.424	4(50)	2(33.33)	0.533	7(38.89)	5(41.67)	0.879

Table 3: Relapse rates and mutation sites in hepatocellular cccDNA P Region

Variable Sites (rt)	Initial treatment (n = 25)			Retreatment (n = 18)			Total (n = 43)		
	Recurrence n = 13 (n, %)	*Non-recurrence n = 12 (n, %)*	*P-value*	*Recurrence N = 9 (n, %)*	*Non-recurrence n=9 (n, %)*	*P-value*	*Recurrence n = 22 (n, %)*	*Non-recurrence n = 21 (n, %)*	*P-value*
278	3(23.08)	5(41.67)	0.319	4(44.44)	6(66.67)	0.64	7(31.82)	11(52.38)	0.172
238	4(30.77)	2(16.67)	0.409	7(77.78)	2(22.22)	0.018	11(50)	4(19.05)	0.033
134	3(23.08)	9(75)	0.009	1(11.11)	1(11.11)	1	4(18.18)	10(47.62)	0.039
222	4(30.77)	4(33.33)	0.891	2(22.22)	1(11.11)	0.527	6(27.27)	5(23.81)	0.795
267	1(7.69)	9(75)	0.001	8(88.89)	7(77.78)	0.527	9(40.91)	16(76.19)	0.028

According to recent studies, mutations in RT gene leads to Nas-resistant mutation sites, which are classified into three categories: primary sites of drug resistance, including rt181, rt184, rt194, rt202, rt204, rt236, and rt250; secondary sites, such as rt80, rt169, rt173, and rt180; and possible compensating sites, including rt84, rt85, rt207, rt213, rt214, rt215, rt229, rt233, rt237, rt238, and rt222. In relation to ADV resistance, main mutations identified are rtA181V/T, rtN236T, and combined mutations of rtA181V/T and rtN236T. Secondary mutations or compensatory mutations, such as L80V/I, N238V/T, V84M, V214A, Q215S, and P237H, have also been observed [3]. Some studies have suggested that a single mutation of rtN238T/D or combined mutation of rtA181V/T and rtN236T may be responsible for ADV resistance [4-7]. Wang F has highlighted that mutation of rtN238T/D/H reduces drug sensitivity of ADV for HBV [8]. *In vitro* experiments have demonstrated that reductions in ADV drug sensitivity are attributed to various mutations, including rtV84M, rtS85A, rtQ215S, rtP237H, and rtN238H/S/T/D [9-11]. However, similar research on a large sample, including 1,865 CHB-infected patients, found that mutations in rt238 were present in 165 individuals, with no significant difference in mutations of rtN238H between treated and untreated patients [12]. Therefore, viral replication and drug sensitivity (LAM or ADV) have little connection with rtN238H mutation but are more closely related to HBV genotype [12].

Another study on CHB-infected patients who underwent combined therapy with LAM and famciclovir revealed presence of gene mutation sites, such as rtL180, rtM204, and rt222T, indicating that virus gene mutations enhance virus replication efficiency, increase virus particle productivity, and alter CTL and B cell epitopes to interfere with immune reactivity, leading to immunity escape and exacerbation of disease progression [13]. Previous evidence suggests that mutations in rtA181T may influence HBV DNA replication and drug sensitivity. Anna et al. [13] observed a patient who experienced hepatic failure after long-term combined therapy with LAM and famciclovir. After analyzing the characteristics of HBV quasispecies, drift was observed in several regions of HBV, including variations in the polymerase gene (rtA222T, rtL180M, rtM204V). Guo et al. [14] confirmed that mutations in rtA222T, rtV207L, rtP237T, and rtI163V are closely related to LAM/ETV resistance, although further evidence from *in vitro* drug sensitivity tests are still needed.

The highest rates of mutation sites were observed in N/H238T, L/F/Q/R267H, V278T, D134E/I, and T222A, both in baseline serum rcDNA and hepatocyte cccDNA during drug withdrawal stage. At recurrence time, highest detectable rates of variation in serum rcDNA were rtN238T, rtT222A, V278T, L/F/Q/R267H, and N248H and predominant variants of rtN238T, rtT222A, V278T, and L/F/Q/R267H were found in the HBV P region. For relapsing patients, detectable rates of T222A in P gene rcDNA and N/H238T in hepatocyte cccDNA were significantly higher compared to non-relapsing patients ($p < 0.05$). In retreatment group, detectable rate of N/H238T in hepatocyte cccDNA P region at time of recurrence was significantly higher in relapsing patients than in non-recurrence ones ($p < 0.05$)., suggesting that mutations of T222A and N/H238T may lead to relapse. However, rtN238T, rtT222A, V278T, and L/F/Q/R267H may be the more stable dominant variants, persisting even after standard LAM therapy. These mutation sites may be difficult to remove, especially for CHB patients who meet drug cessation criteria. At the time of relapse, rtN238T, rtT222A, V278T, and L/F/Q/R267H may be screened and developed into dominant variants.

Mutations at these sites can reduce Nas-sensitivity to some degree, increase HBV replication capacity, and lead to recurrence. There was no statistical difference in V278T and L/F/Q/R267H mutations between recurrence group and non-recurrence group, which may be attributed to relatively shorter follow-up period. Therefore, extending follow-up period is necessary for further studies. Thus, higher variation frequencies of rtN238T, rtT222A, V278T, and L/F/Q/R267H at baseline treatment are associated with a higher risk of recurrence. However, further research with larger sample sizes and additional experiments on cell epitopes and *in vitro* chemo-sensitivity tests are needed to determine concrete relationship between relapse and rtN238T, rtT222A, V278T, and L/F/Q/R267H mutations.

Furthermore, viral breakthrough occurred in two LAM-retreatment patients who subsequently underwent salvage treatment with ADV combined therapy. At baseline level of salvage treatment, rt180 was detected in both patients (100 %), while rt204 was detected in one of the cases (100 %). When cessation criteria for ADV combined therapy were met, rt180 disappeared in both patients, while rt204 was still detected in both patients with detection rates of 79.16 and 66.67 %, respectively. At recurrence time, rt204

was detected in both patients with rates of 100 and 11.11 %. This suggests that in retreatment of drug-resistant patients after receiving standard antiviral therapy and meeting cessation criteria, drug-resistant mutants may not be eliminated.

CONCLUSION

At baseline treatment, higher mutation rates of rtN/H238T, rtT222A, 278T, and L/F/Q/R267H in P region increase risk of relapse. As a result, patients are susceptible to relapse after drug withdrawal. Therefore, retreatment for drug-resistant patients should be prolonged to reduce the risk of recurrence. Large-scale clinical investigation will be required to validate this claim.

DECLARATIONS

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None provided.

Ethical approval

This study was approved by the Ethics Committee of Minzu Hospital of Guangxi Zhuang Autonomous Region (approval no. 20-EC-11).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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