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Character of HBV (hepatitis B virus) polymerase gene rtM204V/I and rtL180M mutation in patients with lamivudine resistance

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Abstract: Objectives: To investigate the relationship between HBV (hepatitis B virus) polymerase gene 180 and 204 sites mutation and lamivudine resistance. Methods: One hundred forty-one patients with lamivudine resistance after lamivudine treatment and 60 chronic hepatitis B patients without lamivudine treatment were enrolled in this study. The serum HBV DNA mutation was analyzed by sequence detection via polymerase chain reaction (PCR). The sequences of the same patient were analyzed before and after lamivudine treatment. Results: One hundred and nine lamivudine resistance patients had HBV YMDD (tyrosine-methionine-aspartate-aspartate) mutation. Among them, 45 patients had rtL180M/M204V mutation (41.28%), 28 patients had rtL180M/M204I mutation (25.70%) and 36 patients had rtM204I mutation (33.02%). There were 6 patients with rtL180M mutation in 32 lamivudine resistance patients. Sixty chronic hepatitis patients without lamivudine treatment had no mutations. Conclusions: HBV mutations, which play an important role in lamivudine resistance usually locate at polymerase gene 204 site; 180 site mutation was also observed in these patients. Evaluation of the anti-virus therapy by surveillance of the two sites mutations is of importance.

Key words: Hepatitis B virus, Lamivudine, YMDD mutant, Sequence analysis
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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem leading to around one million deaths annually worldwide (Lee, 1997). A wide range of clinical manifestations has been established for chronic hepatitis B virus infection, from asymptomatic carriers to severe chronic liver disease, including those with cirrhosis and hepatocellular carcinoma (Chen, 1993; Chu and Liaw, 1997). Lamivudine, an oral nucleoside analogue, inhibits HBV replication (Lai *et al*., 1997; 1998) and can markedly reduce serum HBV DNA levels and normalise alanine aminotransferase (ALT) levels associated with improvement in liver necroinflammatory activity (Lai *et al*., 1998), but the greatest drawback with lamivudine treatment is the emergence of drug-resistant HBV mutants, the mutation of the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the C domain of the HBV DNA polymerase gene (Lai *et al*., 1998; Dienstag *et al*., 1999). The serum HBV DNA mutation of 141 patients with lamivudine resistance and 60 chronic hepatitis B patients without lamivudine treatment was analyzed by sequence detection via polymerase chain reaction (PCR) in order to investigate the relationship between HBV polymerase gene 180 and 204 sites mutation and lamivudine resistance.

MATERIALS AND METHODS

Patients

Two-hundred and one subjects were inpatients and outpatients in our hospital from June 2002 to June 2003. They were 135 men and 66 women, aged 18−53 years. Among them were 141 patients with lamivudine resistance after lamivudine treatment (100

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mg/d) with mean treatment period of one year and a half; and 60 chronic hepatitis B patients without lamivudine treatment. None of the patients was ever treated with interferons and other anti-viral drugs.

Design and synthesis of primers

Upstream primer: 5′ CTCCAATCACTCACCA AC 3′; downstream primer: 5′ GGGTTTAAATGT ATACCCA 3′; sequencing primer: 5′ GTAATTCCC ATCCC 3′. All the primers above were synthesized by Sangon Company, Shanghai (China).

PCR

The amplification reaction contained 1 μl each of 25 μmol/L specific primers, 1 μl 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 4 μl 25 mmol $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promaga) and 5 μl 10×PCR buffer solution. The total volume was brought to 50 μl using ddH_2O. The PCR amplifications were performed in a PTC-200 peltier thermal cycler (MJ Research, USA) under the following conditions: After an initial denaturation for 5 min at 94 °C, samples were subjected to 35 cycles of amplification (94 °C 45 s, 55 °C 45 s, 72 °C 1 min), followed by a final extension of 5 min at 72 °C. The purification of PCR products was performed by QIAquick PCR purification kit according to manufacture's instructions (QIAGEN, USA).

Sequence analysis

Sequence analysis of the PCR products was performed by DYEnamic™ ET dye terminator cycle sequencing Kit (AmershamBioscience) in a MegaBACE™ 500 according to manufacture's instructions; sequence analysis software was used to analyze the results.

RESULTS

Types of HBV polymerase gene 180 and 204 sites mutation

There were 109 lamivudine resistance patients who had HBV YMDD mutation and 141 patients with lamivudine resistance. Among them, 45 patients (45/109; 41.28%) had rtL180M/M204V mutation that played the most important role in YMDD mutation. In addition, 28 patients (28/109; 25.70%) had rtL180M/M204I mutation and 36 patients (36/109; 33.02%) had rtM204I mutation. There were 6 patients with rtL180M mutation in the other 32 lamivudine resistance patients; 60 chronic hepatitis patients without lamivudine treatment had no mutations (Table 1).

Table 1 Types of HBV polymerase gene 180 and 204 sites mutation

Group	Type	*n*
Patients with lamivudine treatment	rtL180M/M204V	45
	rtL180M/M204I	28
	rtM204I	36
	rtL180M	6
	No classification	26
Patients without lamivudine treatment	No mutation	60

Combinatorial mutation rate of M204V/I

All 45 patients with rtM204V mutation also had rtL180M mutation; the rate of this kind of combinatorial mutation was 100%. Among 64 patients with rtM204I mutation, only 28 patients also had rtL180M mutation; the occurrence rate of this kind of combinatorial mutation was 43.75%.

Combinatorial mutation rate of rtL180M

Among 79 patients with rtL180M mutation were 45 patients who had rtM204V mutation, 28 patients who had rtM204I mutation, the combinatorial mutation rate was 56.96% and 35.44%, respectively. In addition, there were only 6 patients who had rtL180M mutation without rtM204V mutation or rtM204I.

Relationship between HBV polymerase gene mutation and lamivudine treatment

Among 141 patients with lamivudine resistance, 115 patients had polymerase gene mutation, either at 204 site or at 108 site. The mutation rate was 81.56%. While in the other group, 60 chronic hepatitis patients without lamivudine treatment did not have any mutations.

DISCUSSION AND CONCLUSION

Chronic hepatitis B virus (HBV) can be treated with a nucleoside analogue, lamivudine (2′,3′-dideoxy-

3′-thiacytidine). In the short term, substantial inhibition of HBV replication can be achieved. However, resistance to lamivudine emerges in approximately 14% of patients after 1 year, rising to 43% after 3 years. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif (codons 203−206 of the reverse transcriptase (rt)), which is part of the catalytic site of the HBV polymerase (Allen *et al*., 1998; Chayama *et al*., 1998; Gutfreund *et al*., 2000; Kirishima *et al*., 2002; Kobayashi *et al*., 2001; Leung, 2002; Yeh *et al*., 2000). Three types of mutations are observed in the polymerase gene. The consensus rt domain numbering system described in Stuyver *et al*.(2001) has been used to describe the variants. There is the M204V associated with L180M (Group I), the M204I alone (Group II) or the M204I with L180M (Group III) (Pillay *et al*., 1998). The L180M and M204V mutations act synergistically to increase resistance to lamivudine (Allen *et al*., 1998).

One hundred and forty-one patients with lamivudine resistance and 60 chronic hepatitis B patients without lamivudine treatment were studied in this work in the Hangzhou area. The serum HBV DNA mutation was analyzed by sequence detection via polymerase chain reaction (PCR). Our results indicated 109 lamivudine resistance patients had HBV YMDD mutation. There were three main types of mutation: rtL180M/M204V, rtM204I and rtL180M/M204I. Among them, 45 patients had rtL180M/M204V mutation (41.3%), which played the most important role in YMDD mutation. In addition, 28 patients had rtL180M/M204I mutation (25.7%) and 36 patients had rtM204I mutation (33.0%).

Our results indicated that rtM204V mutation is always combined with rtL180M; the occurrence rate of this kind of combinatorial mutation was 100%; while rtM204I mutation could exist alone. These results accorded with those of other research groups' (Pillay *et al*., 1998; Allen *et al*., 1998).

Our study revealed that there were 6 patients with rtL180M mutation among the 32 lamivudine resistance patients. This kind of mutation was novel, and requires further study to explore its clinical significance.

Though in this study, 60 chronic hepatitis patients without lamivudine treatment had no mutations, it had been reported that patients without lamivudine treatment could also have mutations; we considered that these contradictory findings had something to do with the fewer samples.

In conclusion, HBV mutations, which play an important role in lamivudine resistance, usually locate at polymerase gene 204 site; 180 site mutation was also observed in the patients. It is of importance to evaluate anti-virus therapy by surveillance of the two sites mutations.

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