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Detection of Lamivudine-Resistant Mutations of HBV DNA Polymerase Gene Using PCR-Direct Sequencing

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Treatment of hepatitis B virus (HBV) with lamivudine is effective in suppressing virus replication and results in reduced inflammatory activity. However the most troublesome problem of lamivudine treatment is the emergence of lamivudine-resistant strains with amino acid substitution in the YMDD motif of DNA polymerase gene during the treatment. The aim of this study was to determine the mutation of YMDD motif (codon 552) and codon 528 in chronic HBV patients with lamivudine therapy using PCR-direct sequencing and to investigate the relationship between lamivudine mediated HBV mutation and HBeAg. HBV DNA was extracted from serum samples of HBV patients and amplified by nested PCR with two sets of primer pairs selected in HBV DNA polymerase gene. Amplified PCR product was analyzed by 2% agarose gel electrophoresis and direct sequencing. HBV mutation was detected in 124 out of 207 samples (60%). Single mutation was 50.8% for M552I, 43.5% for M552V, 5.7% for M552I/V and the L528M mutation was 67.0%. Double mutation was 43.6% for M552V/L528M, 33.1% for M552I/L528(wild type), 17.7% for M552I/L528M and 5.6% for M552I/V/L528M. Serine mutation at YMDD motif (M552S) was not found and the L528M mutation frequently accompanied M552V type. In this study, the typical difference of frequencies for HBV mutation depending on HBeAg was not found. Moreover, the PCR-direct sequencing method used in this study might be a powerful tool for the mutation study in clinical reference laboratories with high volume.

Key words: HBV, Lamivudine, Mutation, HBeAg, PCR direct sequencing

I. INTRODUCTION

Chronic hepatitis B is a common disease with about 350 million Hepatitis B virus (HBV) carriers according to the figures of the World Health Organization (Hanazaki, 2004). The persistence of viremia in patients often leads to progressive liver diseases including cirrhosis and hepatocellular carcinoma. Despite the availability for almost 20 years of safe and effective vaccines against hepatitis B, chronic infection with HBV remains among the ten most common causes of death worldwide (Lok et al, 2000). South Korea is one of the HBV endemic areas where about 5~8% of the total population are infected with HBV (Paik et al, 2001). Interferon has been shown to be beneficial to some patients with chronic HBV infection. However, the overall response rate to interferon alpha is less than 40% (Wong et al, 1993). Recently lamivudine has been used for the therapy of chronic HBV infection. Lamivudine, an enantiomer of b-L-2',3'-dideoxy-

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3'-thiacytidine (3TC), is a potent inhibitor of RNA-dependent DNA polymerase of HBV (Fu et al, 1999). Lamivudine treatment is effective in suppressing HBV replication and decreasing the level of HBV DNA, thereby improving liver function tests and leading to histological improvement (Ning et al, 2005). Prolonged use of lamivudine has been associated with increased emergence of lamivudine-resistant HBV strains with amino acid substitutions in the β-domain (L528M; leucine → methionine) and in the YMDD (tyrosine-methionine-aspartate-aspartate) motif of the C-domain (M552I; methionine → isoleucine, M552V; methionine → valine) of HBV DNA polymerase (Kurihara et al, 2005). The amino acid substitution of YMDD motif inhibits HBV DNA polymerase activity and is the main mutation responsible for resistance to lamivudine treatment. Some reports described that mutation at codon 528 increases HBV replication and drug resistance (Ono et al, 2001). Positive HBeAg and high ALT level could be good candidates for lamivudine therapy (Saintantonio et al, 2000) because lamivudine accelerates the natural serocoversion of HBeAg accompanied by HBV DNA loss. HBeAg could be one of the predictive markers for HBV DNA breakthrough (Tassopoulos et al, 1999). The purpose of this study was to investigate the frequencies of lamivudine resistant HBV mutants associated with HBeAg and to evaluate the usefulness of PCR-direct sequencing for the detection of HBV mutation.

II. MATERIALS AND METHODS

Materials

Serum samples of 207 chronic HBV patients with lamivudine therapy (range of age, 23～59; 153 males and 54 females), from our institute were tested for detecting the lamivudine resistant HBV mutation.

Methods

HBV DNA was extracted from 300 μL of serum sample using PuregeneTM DNA purification kit (Gentra Inc, USA). For amplification of HBV DNA polymerase gene, nested PCR was performed using two pairs of primers described by Chayama et al (1998). PCR amplifications were performed consisting of 4.5 μL of template DNA, 0.5 μM of primer set, 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris-HCl (pH 8.4), 0.5 mM of dNTPs, and 1 Unit of Taq polymerase (Takara, Japan) in a final volume of 20 μL. The amplification conditions including an initial denaturation for 5 min at 94℃, 35 cycles of amplification with denaturation at 94℃ for 1 min, annealing at 52℃ for 1 min, extension at 72℃ for 45 sec, followed by a final extension at 72℃ for 7 min. PCR negative samples by first PCR were re-amplified by 2nd PCR primers using 1.5 μL of the first PCR products under the PCR conditions including an initial denaturation for 5 min at 94℃, 25 cycles of amplification with denaturation at 94℃ for 30 sec, annealing at 60℃ for 30 sec, extension at 72℃ for 30 sec, followed by a final extension at 72℃ for 7 min. The PCR-amplified product (256 bps) of HBV DNA polymerase gene was detected by 2% agarose gel electrophoresis and was purified by using a High Pure PCR product purification kit (Roche Diagnositics, Swiss) according to the manufacturer's instructions. PCR products were subsequently sequenced with a Big-Dye Terminator sequencing kit (Applied Biosystems, USA) by using one of the PCR primers as a sequencing primer. Sequencing reactions were analyzed on the automatic DNA sequencer (Perkin Elmer ABI9600, USA).

III. RESULTS

Lamivudine resistant HBV mutations in chronic HBV infection.

In a total of 207 HBV patient samples, including 153 males and 54 females, the average age of HBV mutant group and wild type group was 41.9 and 33.3 respectively. Lamivudine resistant HBV mutation was found in 124 cases out of 207 HBV patient samples (60.0%) (Table 1). Single mutation of codon 552 was 50.8% for M552I, 43.5% for M552V and 5.7% for M552I/V. The L528M mutation at codon 528 was 67.0% (Table 2). Double

Table 1. The frequencies of lamivudine resistant HBV mutation in chronic HBV patients.

	HBeAg (+) group Number (%)	HBeAg (-) group Number (%)	Total Number (%)
Mutation at Codon 552 or 528	28/50(56.0%)	96/157(61.1%)	124/207(60.0%)
Wild type	22/50(44.0%)	61/157(38.9%)	83/207(40.0%)

Table 2. The frequencies of single mutation of lamivudine resistant HBV.

		HBeAg (+) group Number (%)	HBeAg (-) group Number (%)	Total Number (%)
Codon 552	M552I	14/28(50.0%)	49/96(51.0%)	63/124(50.8%)
	M552V	14/28(50.0%)	40/96(41.7%)	54/124(43.5%)
	M552I/V	0/28(0.0%)	7/96(7.3%)	7/124(5.7%)
Codon 528	L528M	19/28(67.8%)	64/96(66.7%)	83/124(67.0%)

Table 3. The frequencies of double mutation of lamivudine resistant HBV.

	HBeAg (+) group Number (%)	HBeAg (-) group Number (%)	Total Number (%)
M552V/L528M	14/28(50.0%)	40/96(41.7%)	54/124(43.6%)
M552I/L528(WT)	9/28(32.1%)	32/96(33.3%)	41/124(33.1%)
M552I/L528M	5/28(17.9%)	17/96(17.7%)	22/124(17.7%)
M552 I/V/L528M	0/28(0.0%)	7/96(7.3%)	7/124(5.6%)

M552I : Methionine → Isoleucine M552V : Methionine → Valine
M552I/V : Methionine → Isoleucine/Valine L528M : Leucine → Methionine
WT : Wild Type

mutation was 43.6% for M552V/L528M, 33.1% for M552I/L528 (wild type), 17.7% for M552I/L528M and 5.6% for M552I/V/L528M (Table 3). The L528M mutation accompanies frequently M552V type. The M552S (methionine → serine) mutation was not found.

Lamivudine resistant HBV mutations in HBeAg positive group

A total of 207 HBV serum samples were separated into two groups, 50 cases of HBeAg positive group and 157 cases of HBeAg negative group. In HBeAg positive group, HBV mutation was found to be 56.0% (Table 1). Mutations of M552I and M552V showed the same frequency (50.0%). The L528M mutation of codon 528 was 67.8% (Table 2). Double mutation was 50.0% for M552V/L528M, 32.1% for M552I/L528 (wild type) and 17.9% for M552I/L528M. The M552I/V/L528M mutation was not found (Table 3).

Lamivudine resistant HBV mutations in HBeAg negative group

In HBeAg negative group, HBV mutation was found to be 61.1% (Table 1). Single mutation of codon 552 was 51.0% for M552I, 41.7% for M552V and 7.3% for M552I/V. The L528M mutation of codon 528 was 66.7% (Table 2). Double mutation was 41.7% for M552V/L528M, 33.3% for M552I/L528 (wild type), 17.7% for M552I/L528M and 7.3% for M552I/V/L528M (Table 3).

Electropherograms of lamivudine resistant HBV mutations in codon 552 and 528 using PCR-direct sequencing are presented in Fig. 1.

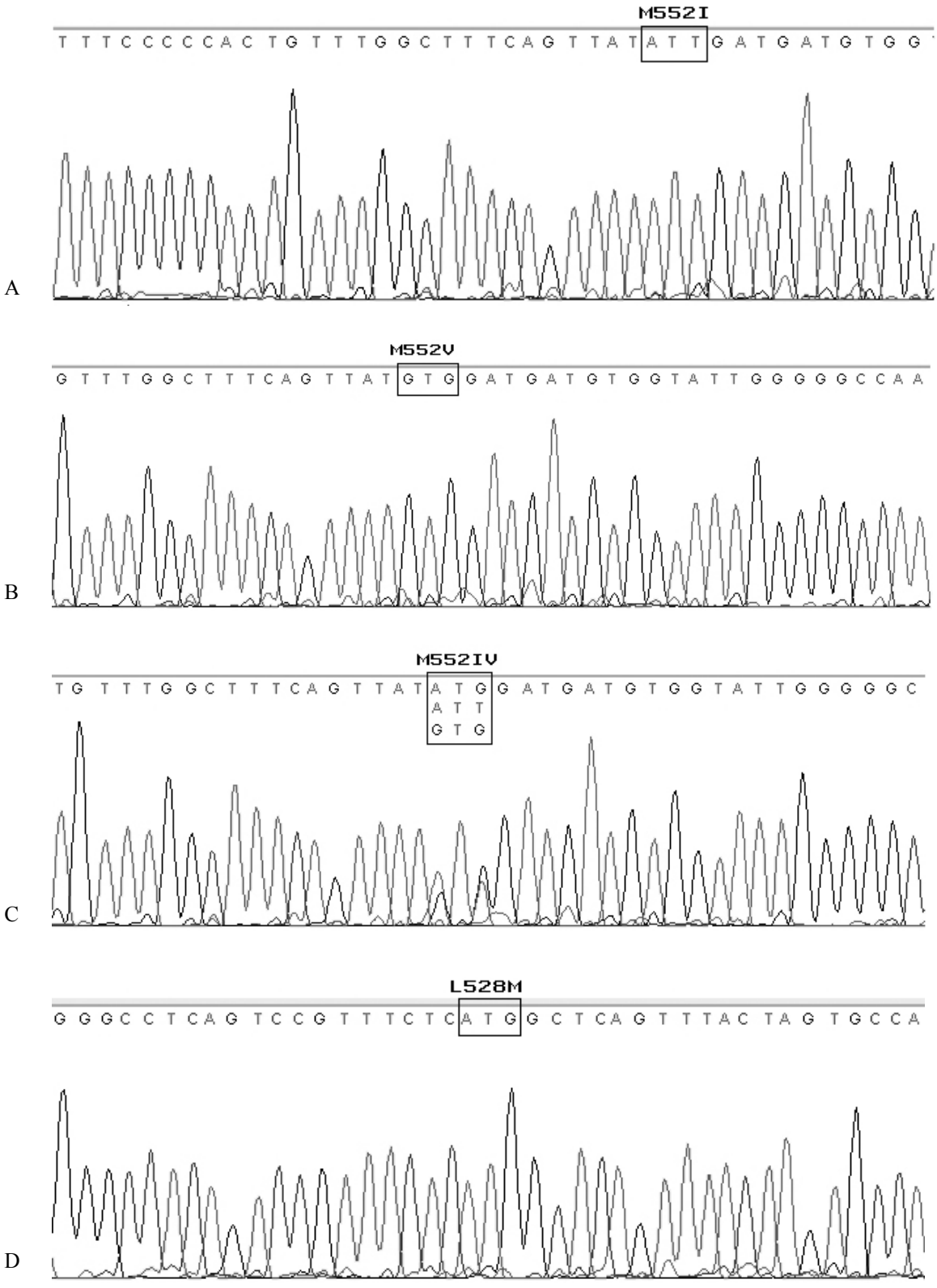

Fig. 1. Electropherograms of lamivudine-resistant HBV mutants at codon 552(a~c) and codon 528(d).

IV. DISCUSSION

Lamivudine inhibits HBV replication by suppressing the terminator and HBV DNA polymerase activity in the wild type YMDD motif. Lamivudine-resistant HBV harbor M552I or M552I/V mutations in the C-domain of the HBV polymerase gene. The mutation of methionine to isoleucine or valine in the YMDD motif of HBV reverse transcriptases confers resistance to lamivudine because the side groups of isoleucine and valine of the YMDD mutants sterically prevent lamivudine from appropriate configuring into the nucleotide binding site of the reverse transcriptase (Allen et al, 1998). Although lamivudine was approved for the treatment of patients with chronic HBV infection, short-term treatment is usually insufficient to clear the virus. Moreover, long-term treatment is associated with the development of drug-resistant HBV in 14-75% of patients. The risk of emergence of lamivudine resistance increases with the duration of treatment (Chayama et al, 1998). Ono et al (2001) reported that L528M mutation in the β-domain of HBV polymerase gene not only restores the replication competence of C-domain mutants, but also increases resistance to nucleoside analogues. Therefore the determination of mutation of L528M of β-domain has important clinical significances.

The clinical frequency of mutations in HBV resistant to lamivudine was 60% in this study (Table 1). The average age of HBV mutant group was higher than that of the wild type group (41.9 vs 33.3). On the other hand, Kim et al (2002) reported that the average age of the mutant group was significantly lower than that of the wild type group in the Korean population. With regard to the single mutations of codon 552, M552V and M552I was 43.5% and 50.8%, respectively (Table 2). However, Allen et al, (1998) reported that the M552V mutation was found more than 90% in Whites. Kim et al, (2002) reported that mutations of M552V was 21.0% and M552I was 68.0% in Koreans. The most frequent double mutant type of this study was M552V/L528M and it was correlated well with reports of Whites (Benhamou et al, 1999; Neisters et al, 2002) and Japanese (Chayama et al, 1998; Ono et al, 2001). Kim et al, (2002) reported that HBeAg positive group showed a higher mutation rate than HBeAg negative group in the Korean population. However we did not find any critical differences of mutation frequencies with HBeAg and this result showed a good correlation with the studies from Italy, England and other European countries (Tassopoulos et al, 1999; Lok et al, 2000; Saintantonio et al, 2000). Some discrepancies were found when our data were compared with other reports as above. It might come from several factors including detection methods, number of samples, HBV genotypes in different ethnic groups and clinical therapeutic status of HBV patients. From this study, single β-domain mutant L528M frequently accompanies mutants of M552V or M552I/V and it was similar with the previous reports by other study groups (Bartholomew et al, 1997; Kim et al, 2002). Serine mutation at codon 552 described by Hubert et al, (2002) was not found in chronic HBV infection. In this study, no relevance was found that HBeAg was associated with the frequency of HBV mutation and mutant types, single or double mutations at codon 552 and 528 (Table 1～3).

Several techniques have been used to study the viral polymorphism, including type-specific PCR, PCR-SSCP (single strand conformation polymorphism), PCR-RFLP (restriction fragment length polymorphism) (Ning et al, 2005), oligonucleotide chips (Heo et al, 2004) and PCR and hybridization with specific probes. DNA chip using oligonucleotide is a new technology for the detection of point mutation, however it requires a scanner for data analysis and sometimes carry-over could be a problem to solve(Ning et al, 2005). In this study, we analyzed lamivudine-resistant HBV mutants by PCR-direct sequencing method which is considered as the gold standard for the mutation study.

V. CONCLUSION

The appearance of lamivudin-resistant HBV mutation in HBV patients presents an important clinical significance for HBV patients. Clinicians might consider changing the drug when lamivudine-resistant mutation is found in HBV

patients. In this study, lamivudine-resistant HBV mutations were determined using PCR-direct sequencing in HBV patient sera. The Sequencing technique showed clear discrimination of HBV mutant types in codon 522 and 528 and it could be a useful tool for the lamivudine-resistant HBV mutations in reference laboratories with high sample volume. Moreover the mutation frequencies of lamivudine-resistant HBV should be a useful information for the follow up study of patient and for changing therapeutic drugs. Further correlation studies between lamivudine resistant HBV mutant types and clinical data of patients, including HBV-DNA, HBeAg and liver function tests, are needed for the treatment and prognosis of chronic HBV patients.

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국문 요약

최근 만성 B형간염의 치료에 B형간염 바이러스 복제를 저해하여 감염력을 약화시키는 lamivudine이 많이 사용되고 있다. 그러나 이 약제를 장기간 복용할 경우 B형간염 바이러스 DNA polymerase 유전자의 YMDD motif에 아미노산 치환을 일으켜 lamivudine 저항성 B형간염 바이러스가 나타나는 점이 문제시 되고 있다. 본 연구의 목적은 lamivudine 치료를 받은 만성 B형간염 환자에서 PCR-direct sequencing 법을 이용하여 B형간염 바이러스 DNA 중합효소 유전자의 YMDD motif(codon 552)와 codon 528에서 돌연변이 출현빈도를 구하고, HBeAg과의 관련성을 보고자 하였다. 방법은 만성 B형간염 환자의 혈청에서 DNA를 추출하고 DNA 중합효소 유전자의 codon 552와 528을 포함하는 부위에서 선택한 두 쌍의 primer를 이용하여 nested PCR을 실시하였다. 증폭된 PCR 산물은 2% agarose gel에서 전기영동을 한 후, 자동염기서열분석기를 이용하여 sequencing을 실시하였다. 총 207명 중에서 돌연변이는 124명(60%)에서 발견되었으며, 남, 녀에서 차이는 발견되지 않았고, 돌연변이군에서 비돌연변이군에 비해 평균나이가 약간 높게 나타났으나 유의성은 없었다. Codon 552에서 단일돌연변이로는 M552I(50.8%)가 가장 높게 나타났고, 다음으로 M552V(43.5%), M552I/V(5.7%)의 순서로 나타났다. Codon 528에서는 67.0%의 L528M 돌연변이가 발견되었다. Codon 552와 codon 528에서 동시에 발생한 중복돌연변이로는 M552V/L528M(43.6%)이 가장 높게 나타났고, 다음으로 M552I/L528(33.1%) 그리고 M552I/L528M(17.7%)의 순으로 나타났다. Codon 552에서 serine 돌연변이(M528S)는 발견되지 않았으며, L528M은 M552V 돌연변이와 거의 동시에 검출되었다. 본 연구에서 만성 B형간염환자에서 HBeAg의 유무와 lamivudine 돌연변이율과의 상관성은 발견되지 않았으며, PCR-direct sequencing법은 고가의 자동염기서열분석 장비와 숙련된 기술자가 필요하다는 문제점은 있으나, 검체 수가 많은 큰 임상검사실에서는 활용성이 클 것으로 판단된다. 향 후 lamivudine으로 인한 HBV 돌연변이형과 환자의 임상결과의 관련성에 대한 연구가 추가적으로 실시되면, lamivudine을 복용하는 만성 HBV 환자의 치료와 예후에 유용할 것으로 사료된다.