Demonstration of mutations conferring resistance to lamivudine in liver tissue of chronic hepatitis B patients under lamivudine therapy*

İsmail Yaşar AVCI[1],[**], Can Polat EYİGÜN[1], Emine GÜNAL[1], Ayhan KUBAR[2], Hanefi Cem GÜL[1], Ömer COŞKUN[1], Hakan ERDEM[3], Levent GÖRENEK[3], Bülent Ahmet BEŞİRBELLİOĞLU[1]

[1]Department of Infectious Diseases and Clinical Microbiology, Gülhane Military Medical Academy, Ankara, Turkey
[2]Department of Virology, Gülhane Military Medical Academy, Ankara, Turkey
[3]Department of Infectious Diseases and Clinical Microbiology, Gülhane Military Medical Academy,
Haydarpaşa Training Hospital, İstanbul, Turkey

Aim: To demonstrate the genotypic lamivudine resistance in liver tissue of patients with negative serum hepatitis B virus (HBV) DNA under long-term lamivudine therapy.

Materials and methods: Fourteen patients (10 patients with elevated alanine aminotransferase and 4 patients under long-term lamivudine therapy for severe liver disease) with undetected serum HBV DNA were included in the study. A liver biopsy was performed for each patient, and HBV DNA extracted from liver tissue was analyzed with TaqMan real-time polymerase chain reaction.

Results: Mutations conferring lamivudine resistance were shown for all 14 patients in their liver tissue-extracted HBV DNA. One patient had rtM204V, 4 patients had rtL180M, 2 had rtL180M + rtM204V, 4 had rtL180M + rtM204I, and 3 had rtL180M + rtM204I + rtM204V substitutions.

Conclusion: Analyzing mutation in liver tissues of these patients might help demonstrate resistance earlier and prevent delaying of therapeutic modification. Therefore, all liver biopsy specimens of patients with severe liver fibrosis should be analyzed using the molecular technique.

Key words: Mutation, liver tissue, HBV DNA

1. Introduction

Hepatitis B virus (HBV) causes a vaccine-preventable disease and mass vaccination programs can successfully reduce the prevalence of infection (1). However, there are still an estimated 350–400 million people infected with HBV all over the world. HBV infection has been associated with long-term mortality and morbidity due to its complications. Approximately 15%–40% of these HBV-infected people develop liver cirrhosis, hepatic decompensation, or hepatocellulary carcinoma. In all forms of HBV infection, quality of life is negatively affected. Additionally, HBV causes a million deaths each year (2–5). The main purposes of the treatment of chronic HBV-infected people are to suppress viral replication, to normalize alanine aminotransferase (ALT) levels, to limit progressive liver disease, and to prevent hepatic decompensation or development of hepatocellulary carcinoma (6–9).

The introduction of nucleoside analogues provided ALT normalization, decrease or clearance in viral load, HBeAg disappearance or seroconversion, and improvement in liver histology. Unfortunately, discontinuing therapy caused relapses in many patients or increased the requirements for long-term therapy. Long-term therapies touched off the development of resistant mutations (10–12). Lamivudine is associated with the emergence of mutations in the YMDD motive (tyrosine, methionine, aspartate, aspartate) of HBV DNA polymerase domain C (rtM204V/I) and with upstream compensatory mutations in polymerase domains A and B (rtL180M) that collectively reduce treatment efficacy (13). Additionally, the YSDD (rtM204S) mutation may cause biochemical and virological breakthrough (14). In case resistance to lamivudine develops, one of various individualized rescue strategies can be selected (15).

The aim of this study is to demonstrate genotypic lamivudine resistance in liver tissue of patients with elevated serum ALT levels whose serum HBV DNA cannot be detected under lamivudine therapy or patients under long-term lamivudine therapy for severe liver disease whose serum ALT levels are within normal limits and serum HBV DNA is not detected.

2. Materials and methods

This study was carried out the Gülhane Military Medical Academy Department of Infectious Diseases and Clinical Microbiology in 2007 and 2008.

A total of 14 patients (all males; 10 patients whose serum ALT levels were elevated but whose serum HBV DNA could not be detected under lamivudine therapy and 4 patients under long-term lamivudine therapy for severe liver disease whose serum ALT levels were within normal limits and for whom HBV DNA was not detected) were included in the study. Serum biochemical analyses were performed by autoanalyzer according to the manufacturer's instructions (Sigma Co., USA). Serum hepatitis B surface antigen (HBsAg), antibody (anti-HBs), hepatitis B e antigen (HBeAg), and antibody (anti-HBe) were analyzed by microparticle enzyme immunoassay (AxSym System, Abbott, USA). A liver biopsy was performed on each patient after obtaining their informed consent. Histopathology of the liver was evaluated using the Modified Knodell Histopathological Activity Index.

The fibrosis scores of the 4 patients with severe liver histology were 4, 5, 5, and 6/6, respectively. As they were under lamivudine therapy for a long time, they were expected to develop lamivudine resistance although their ALT levels were within normal limits and HBV DNA was not detected.

Serum HBV DNA and HBV DNA extracted from liver tissue were analyzed with TaqMan real-time polymerase chain reaction (PCR) using forward 5′-ATCCTCACAATACCRCAGAGT-3′ and reverse 5′-CAAATGGCACTAGTAAACTGAGC-3′ primers and TaqMan Probe FAM ACTCGTGGTGGACTTCTCTCAATTTTC BHQ-1 as described by Kubar et al (16). The minimum HBV DNA detection limit of the method used was 10^2 copies/mL. This method was conducted using gene sections created by cloning the above mentioned primers with the TOPO-TA Cloning Kit (Invitrogen, USA) as a plasmid. World Health Organization standards were used to set viral load detection limits.

Lamivudine resistance was investigated with the primers and probes given below (in-house):

HBVCOP1	CGACGGAAAYTGCACYTGTA (common primer),
LAM528MUTP2	TGGCACTAGTAAAYTGAGCCTTA (primer for codon 180),
LAM552IMUTP2	CCCCCAAWACCACATCATAAT , and
LAM552VMUTP2	CCCAAWACCACATCATCCTCT (primers for codon 204), 528PR
FAM	AAACGGRCTRAGGCCCACTCC BHQ (probe for codon 180),552PR
FAM	AAAGCCAWRCARTGGGGGAAAGCC BHQ (probe for codon 180).

3. Results

The patients were 22 to 68 years old (median: 41.5 years) and their ALT levels were 13 to 104 IU/L (normal range: 5–40 IU/L, median: 39.5 IU/L). They were under lamivudine therapy for periods in the range of 1 to 10 years (median: 5 years). Five patients were HBeAg positive. Serum HBV DNA from these patients did not return positive results for breakthrough, despite multiple tests, and so we could not prove them to be lamivudine-resistant. In HBV DNA extracted from liver tissue, mutations conferring lamivudine resistance were shown for all 14 patients. One patient had rtM204V, 4 patients had rtL180M, 2 had rtL180M + rtM204V, 4 had rtL180M + rtM204I, and 3 had rtL180M + rtM204I + rtM204V substitutions. HBV DNA measurement and types of substitutions for each patient are shown in the Table.

4. Discussion

Lamivudine has been widely used in chronic hepatitis B therapy because of its advantages of infrequent side effects, high patient-tolerability, and low cost, despite high occurrences of resistance and an unpredictable duration of therapy.

Though recently developed molecular techniques provided rapid and accurate quantitation of HBV DNA, standardization and technical problems, fluctuation of serum HBV DNA, and limited or no detection of serum HBV DNA in patients with advanced liver disease because of insufficient hepatocyte reserve can be misleading in diagnostic and therapeutic approaches.

In our study we have shown lamivudine resistance in liver tissue-extracted HBV DNA of patients with elevated ALT and negative serum HBV DNA, as well as patients under long-term lamivudine therapy with normal ALT and negative serum HBV DNA. All patients were lamivudine-resistant in terms of liver tissue-extracted HBV DNA. This implies that genotypic resistance develops earlier than virological and biochemical breakthrough.

In patients for whom virological breakthrough cannot be detected due to poor sensitivity of the assays, the mutant virus continues to replicate. As a result, ALT levels are elevated and biochemical breakthrough develops.

Resistance is defined as the increase in the baseline HBV DNA level after antiviral treatment decreased the baseline levels, provided that the sensitivity of the method used for determining viral load is high. However, if the method used is not sensitive enough, resistance cannot

Table. Liver-extracted HBV DNA level and substitutions for each patient.

Patient	Liver-extracted HBV DNA, copies/mg	Substitutions
1	3800	rtL180M
2	61,000	rtL180M, rtM204V
3	7,600,000	rtL180M, rtM204I
4	230,000	rtL180M, rtM204I, rtM204V
5	6300	rtL180M, rtM204I, rtM204V
6	730,000,000	rtL180M, rtM204V
7	130,000	rtL180M
8	580,000	rtM204V
9	580,000	rtL180M, rtM204I, rtM204V
10	380,000	rtL180M
11	60,000,000	rtL180M
12	73,000,000	rtL180M, rtM204I
13	52,000,000	rtL180M, rtM204I
14	720,000	rtL180M, rtM204I

be detected, and the mutant strain continues to replicate and gain dominance. After a while, as a natural result of liver cells being infected with the mutant strain, serum ALT level increases and biochemical breakthrough and/or acute exacerbations may occur (17–19).

Delayed detection of virological breakthrough has a risk of decompensation or fulminant liver failure for patients with severe fibrosis and for cirrhotic and liver-transplanted immunosuppressed patients.

Though Thompson et al. reported that serum HBV levels were expected to correlate with intrahepatic HBV DNA (20), in our study serum HBV DNA analyses yielded negative results repeatedly for patients with high levels of liver-extracted HBV DNA. We think that for viruses not having direct cytopathic effects, correlations between serum and tissue extracted viral load should not exist.

We used the TaqMan real-time PCR mutation system to analyze mutations. Sequence analysis could also be used to detect different kinds of mutations; the system that we used also had a sequence detection system. That said, sequence analysis has limited sensitivity for detecting mutant viruses when the mutant virus composes less than 25% of the total viral pool (21). However, sequencing could be used to confirm the mutation detected with the TaqMan real time PCR system. In that case, the study would be considered a system analysis study.

Our study revealed that monitoring serum ALT and serum HBV DNA may not be sufficient for patients with elevated ALT and negative HBV DNA under lamivudine therapy or for patients with severe liver histology and normal ALT. Analyzing mutations in the liver tissues of such patients might help us demonstrate lamivudine resistance earlier and prevent the delay of therapeutic modifications. Therefore, all liver biopsy specimens of patients with severe liver fibrosis should be analyzed using molecular technique.

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