Emergence of a Novel Mutation in the FLLA Region of Hepatitis B Virus during Lamivudine Therapy

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The emergence of resistance to lamivudine has been one of the major stumbling blocks to successful treatment and control of hepatitis B virus (HBV) infections. The major mechanism of resistance has been attributed to the alteration in the YMDD motif of the HBV polymerase due to an amino acid change of rtM204 to V/I and an accompanying rtL180M conversion. A novel mutation pattern in a patient having clinical breakthrough under lamivudine therapy was discovered. The mutant had a rtL180C/M204I genotype and was detected after 2 years of therapy with lamivudine. To characterize this novel variant, site-directed mutagenesis was performed using a vector construct containing the HBV genome. Transient transfection studies in human hepatoma cells with HBV carrying the new mutant demonstrated that the rtL180C/M204I mutant was resistant to lamivudine up to 10 μM. The resistance profile was comparable to that of the previously reported rtL180 M/M204I-containing virus. These observations were further confirmed by generation of stable cultures transfected with the mutant virus.

Hepatitis B virus (HBV) is a pathogen that afflicts over 350 million people with liver disease worldwide (27). The persistence of the infection can have devastating consequences for the infected persons, as the natural progression of the disease culminates with cirrhosis of the liver and hepatocellular carcinoma. Until recently, only two treatment options, alpha interferon and lamivudine, were available for the management of HBV. Recently, adefovir dipivoxil and entecavir were approved for the management of hepatitis B, whereas lamivudine therapy is still employed by the virus to circumvent inhibition of the viral replication machinery. The present studies are a continuation of our efforts to identify mechanisms underlying clinical resistance to lamivudine. A novel genotype with the rtL180C change accompanied by rtM204I in the YMDD motif of HBV polymerase was identified during the course of lamivudine treatment. To the best of our knowledge, this is the first report of such an HBV variant in the clinic. We have characterized a cloned virus containing this YIDD change and related mutation (rtL180C/M204I) in the polymerase region focusing on the in vitro susceptibility to lamivudine.

MATERIALS AND METHODS

Patient history. A 68-year-old Caucasian male individual presented initially with Kaposi's sarcoma for which chemotherapy was administered. Subsequently the patient had to undergo surgery for a broken leg. After two years, the patient was also diagnosed with diabetes mellitus. The blood tests on following visits were positive for HBsAg. Liver biopsy revealed chronic active hepatitis with a histological activity index of 6, stage 1. The patient was initiated on alpha interferon therapy (6 mU three times a week) for 4 months and subsequently on lamivudine at a dose of 150 mg/day for about 3 years and 4 months. The study was conducted in accordance with the ethical guidelines of the 1995 Declaration of Helsinki. Informed consent from the patient was obtained for use of samples for the study. Protocols used in the study were approved by the Ethics Committee of the Ankara Medical School, Ankara, Turkey.

Characterization of serum HBV from the patient. HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HCV, and anti-HIV were determined by the microparticle enzyme immunoassay and anti-HDV by the enzyme-immunoassay method (Abbott Laboratories, Illinois). HBV-DNA levels were determined by using a commercially available liquid-hybridization assay, having a detection limit of 5 pg/ml for viral DNA (Digene), according to the instructions provided with the kit. All serum samples were stored at −70°C.

Sequence analysis of HBV during the course of study. The HBV DNA from the serum samples was isolated by digestion with proteinase K followed by phenol/chloroform extraction and ethanol precipitation. PCR amplification and sequencing were performed using published methodology (7).
cells transfected with the rtL180C/M204I mutant HBV vector. The DNA was virus was performed by Southern analysis of the HBV DNA obtained from the transfectants were also analyzed for hepatitis B surface antigen (HbsAg). The assay was performed using the AUSZYME monoclonal diagnostic kit (Abbott Laboratories, Chicago, IL).

Activity. This enzyme activity was determined according to the protocols supplied by the manufacturer (Promega Life Sciences, Madison, WI). Culture supernatants grown in the absence of tetracycline were collected and DNA isolation was performed. PCR amplification of the polymerase region was conducted using HBV-specific primers.

TABLE 1. Clinical and virological characteristics of patient with L180C/M204I HBV mutation

<table>
<thead>
<tr>
<th>Diagnosis and treatment</th>
<th>Clinical visit date (mo.day.yr)</th>
<th>HBV mutation</th>
<th>Liver enzymesa</th>
<th>HBV DNA (pg/ml)</th>
<th>Biopsy</th>
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<tr>
<td>Diagnosed with Kaposi’s sarcoma</td>
<td>1985</td>
<td></td>
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<td>Received chemotherapy for 1 yr</td>
<td>1987</td>
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<td>Received 7 units of blood during surgery for broken leg</td>
<td>1992</td>
<td></td>
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<td></td>
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<td>1997b</td>
<td></td>
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<tr>
<td>Liver biopsy done</td>
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<td>04.30.2002</td>
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Characteristics of HBV in patient serum. At the initial presentation, the patient had >2,000 pg/ml of HBV DNA as determined by the Digene test. Blood tests revealed positivity.

RESULTS

Characteristics of HBV in patient serum. At the initial presentation, the patient had >2,000 pg/ml of HBV DNA as determined by the Digene test. Blood tests revealed positivity.

Construction of rtM204I and rtL180C/M204I HBV mutants. The single and double mutants of HBV were generated by conversion of the amino acid residue at position 204 to isoleucine and at 180 to cysteine or methionine. The vector designated pUHD containing the HBV expression cassette driven by tetracycline-regulated promoter was used for mutagenesis studies (13, 14). This vector has HBV of genotype ayw and contained valine in place of methionine at position 204 (YVDD motif). Site-directed mutagenesis was performed to substitute ATT in place of GTG in the DNA coding for the YVDD motif and TGT in the place of CTG in the coding domain for the FLLA region using the QuickChange kit (Stratagene, Cedar Creek, TX). PCR amplification of the DNA polymerase region of the mutant vectors derived from the site-directed mutagenesis was performed (21). The PCR products were resolved on an agarose gel and the amplified DNA was purified and sequenced to confirm the presence of the YIDD motif.

Generation of mutant HBV in hepatoma cells. Transient transfections were performed as described earlier (21). Plasmids carrying the various HBV variants generated by site-directed mutagenesis were cotransfected with a plasmid designated pUHD, carrying the neomycin resistance gene, and a second vector designated pat0303 containing the HBV expression cassette driven by tetracycline-regulated promoter was used for mutagenesis studies (13, 14). This vector has HBV of genotype ayw and contained valine in place of methionine at position 204 (YVDD motif). Site-directed mutagenesis was performed to substitute ATT in place of GTG in the DNA coding for the YVDD motif and TGT in the place of CTG in the coding domain for the FLLA region using the QuickChange kit (Stratagene, Cedar Creek, TX). PCR amplification of the DNA polymerase region of the mutant vectors derived from the site-directed mutagenesis was performed (21). The PCR products were resolved on an agarose gel and the amplified DNA was purified and sequenced to confirm the presence of the YIDD motif (GTG to ATT) and CTG to TGT at position 180.

Characterization of the stable cell cultures. Cell cultures derived from the G418-resistant colonies were characterized for the expression of HBV. Culture supernatants grown in the absence of tetracycline were collected and DNA isolation was performed. PCR amplification of the polymerase region was conducted using HBV-specific primers.

Analysis of the effect of lamivudine on mutant viruses from stable cell cultures. Stable cells carrying rtM204I and rtL180C/M204I were seeded at a density of 10^5 cells per 25-cm^2 flask in culture medium containing tetracycline. On the fifth day, fresh medium with appropriate concentrations of lamivudine was added. The experiment was terminated on the eighth day. Cells were harvested and processed for HBV DNA from viral particles as described above for transient transfections. An aliquot of the purified DNA was analyzed by real-time PCR for HBV specific DNA (7). HBV DNA standards with predetermined copy numbers (genome equivalents) ranging from 8 \times 10^2 to 8 \times 10^7 were incorporated in the experiment to monitor the copy numbers of the unknown samples as well as to determine the efficiency of PCR amplification.

RESULTS

Characteristics of HBV in patient serum. At the initial presentation, the patient had >2,000 pg/ml of HBV DNA as determined by the Digene test. Blood tests revealed positivity.
HBsAg and HBeAg but were negative for anti-HBe, anti-HBs, and anti-Delta. No indications of presence of HIV and HCV were noted as determined by tests specific for these viruses. Lamivudine treatment over a period of 2 years did lower the viral load to <5 pg/ml. However, 22 months after this observation the viral load increased to 1,000 pg/ml (Table 1).

Emergence of the mutant HBV. The initial observation of change in the HBV polymerase sequence was noted in the

![FIG. 1. Evolution of the novel HBV variant from the wild-type virus. Virological assays and determinations were performed as described in Materials and Methods.](http://aac.asm.org/)

![FIG. 2. DNA sequence alignment of pol region of mutant HBV generated by site-directed mutagenesis. Generation of the plasmids and PCR was performed as described in Materials and Methods. The sequence of the pol region flanking the FLLA region and the YMDD motif is depicted.](http://aac.asm.org/)
FLLA region. The change detected was a conversion of CTG to TTG, which is a polymorphism. Subsequently, a two-nucleotide mutation was noticed, transforming the CTG codon (leucine) to TGT (cysteine). Simultaneously, the YMDD motif was altered from ATG to ATT, resulting in the rtL180C/M204I genotype (Fig. 1).

Confirmation of the mutant HBV generated by site-directed mutagenesis. The mutant HBV vectors generated by site-directed mutagenesis showed the presence of the resistance mutations. Plasmid vectors rtL180C, rtM204I, and rtL180C/M204I were successfully generated. The sequence analysis using the forward primer showed the presence of rtM204I (nucleotide change to ATT) as well as rtL180C (conversion of CTG to TGT) (Fig. 2). There were no additional mutations in the polymerase region other than the ones introduced by site-directed mutagenesis. The sequence was also confirmed by reverse sequencing.

Susceptibility of rtL180C/M204I HBV in transfected cells to lamivudine. The supernatants of the cultures transiently transfected with the various vectors showed the production of HBsAg, indicating the formation of viral particles in the transfected cells. Real-time PCR analysis of the DNA from control and lamivudine-treated transfected cells showed that the levels of HBV DNA were not significantly inhibited (Fig. 3). The rtM204M wild type showed sensitivity to lamivudine at 10 µM, whereas the rtL180C/M204I mutant was resistant. The pattern of the resistance, under these conditions, was similar to that of the rtM204I variant. The resistance pattern remained the same when normalized either to β-galactosidase activity or levels of HBsAg. Transfection studies of HBV with alteration only in the FLLA region (rtL180C/M204M and rtL180M/M204M) in Huh-7 cells showed that L180C and L180M are resistant to lamivudine whereas the wild-type HBV (rtL180L/M204M) was sensitive under the conditions of the assay (Fig. 4).

The resistance phenotype of the virus containing rtL180C/M204I was further confirmed by visualization of the replicative intermediates on Southern blots hybridized to 32P-labeled HBV-specific probes. β-Galactosidase activity was also detected in the cell extracts of the transfected cells, confirming the uptake and expression of the transfected vectors in the Huh-7 cells. The data, normalized to intracellular β-galactosidase activity, confirmed the lack of inhibition of antiviral activity on the novel HBV variant by lamivudine (Fig. 5A and B).

Stable cell cultures containing the rtM204I and rtL180C/M204I mutations that were selected with G418 were positive for HBsAg in the culture supernatants (data not shown). DNA purified from the supernatants when amplified with primers specific for the DNA polymerase region, showed products consistent with that expected for HBV (Fig. 6A). Further confirmation of the resistance pattern of the novel mutant HBV genotype is depicted in the HBV response to lamivudine in the stable transfectants. Repeated treatments of lamivudine to cultures expressing the mutant virus showed no inhibitory activity.
on the DNA replication up to 10 μM. The data from the quantification by real-time PCR of HBV DNA from untreated cells and cells treated with lamivudine demonstrated lack of inhibitory effect (Fig. 6B).

DISCUSSION

Currently, alpha interferon, lamivudine, adefovir dipivoxil, and entecavir are the only four treatment modalities used for the management of HBV infection, although other anti-HBV agents are now in Phase 3 clinical trials (4, 23). Long-term use of lamivudine in the clinic has generated a plethora of information with respect to the clinical response, as well as the emergence of resistant phenotypes of HBV (18). While there are some similarities with respect to the mutations in the active site of both the HBV and HIV polymerases selected by lamivudine, similar resistant variant of HBV exhibit some unique mutations outside the catalytic region. It is postulated that these changes are paramount for the virus empowering it with the advantage required for its survival in the presence of the drug. The most common change observed in the FLLA region is the conversion of amino acid leucine (L) to methionine (M) at position 180.

The present study stems from the observation in the clinic of a novel mutation in an individual undergoing lamivudine therapy for HBV infection. The virus exhibited a gradual progression from wild type to a rtM204I variant in the pol gene. Also accompanying the change in the YMDD motif was the conversion of L (leucine) to C (cysteine) at the amino acid position 180, leading to FLCA instead of FLLA. This amino acid change did not create a stop codon in the overlapping surface antigen sequence open reading frame.

We have for the first time documented the emergence and persistence of a novel rtL180C/M204I variant of HBV during lamivudine therapy. The mutation rtM204I has been described earlier to appear in response to lamivudine monotherapy. However, some studies have shown the preponderance of rtM204I in the second year of treatment in comparison to rtM204V during the first year (8, 9). The conversion of methionine (M) to valine/isoleucine (V/I) at position 204 in the YMDD motif renders HBV resistant to lamivudine (1). The virus with rtM204V can also acquire rtL180M change and these are the most common genotypes seen in the clinic in response to long term lamivudine therapy. Our studies show that in addition to the above changes that confer resistance, novel amino acid changes such as leucine to cysteine could culminate in the same end point, which is drug-resistant HBV.

We have reported recently an rtM204S mutation that resulted in virus resistant to lamivudine by generating stable transfecants that express HBV with the YSDD motif (7). A similar observation was also made by other researchers in the clinic (20). Also, unique mutations, rtA222T and rtL336V, accompanied with mutations in the basal core promoter, core, surface antigen, and X protein regions have been reported (3).

The fluctuations in the viral load in the patient seen during the course of treatment conform to characteristic pattern observed in the clinic during antiviral therapy. There is a steady and profound initial response to the drug due to the sensitivity of the wild-type virus followed by the slow but marginal increase in the viral load. The latter phase is contributed by the emerging resistant variant(s). Even though this variant population does not reach the initial wild-type levels, they present a formidable barrier to successful treatment due to their recalcitrance to the antiviral used. The low replicative capacity of

FIG. 5. A. Replicative intermediates of rtL180C/M204I HBV from transiently transfected Huh-7 cells. Southern analysis of HBV DNA from rtL180C/M204I HBV transfections was performed as described in Materials and Methods. OC: open circular HBV DNA; DS: double-stranded HBV DNA; SS: single-stranded HBV DNA; NT: nontransfected. The positions of the size markers of HindIII-digested lambda markers (4 kb and 2 kb) are indicated. B. β-Galactosidase activity from rtL180C/M204I HBV transient transfection. β-Galactosidase activity from the cells cotransfected with a vector carrying the β-gal gene was performed as described in the Materials and Methods section.
some of these mutants may render them inept to achieve high viral loads (7, 23).

Our studies also underscore the challenges faced in tackling HBV resistance. Even though variants such as rtM204S as well as the one we report in this manuscript are not as frequent as rtL180M/M204V, they could exist in subjects as minor populations and contribute to the clinical resistance. Based on the proposed model for HBV and the interaction of lamivudine with the polymerase, it is suggested that the compensatory nature of rtL180M mutation is due to the interaction of the methionine to its neighboring amino acids (10) and steric hindrance (28). Based on the minimal energy fit of this model, leucine does not cause this interference in stacking. Since both methionine and cysteine are sulfur-containing amino acids, with similar side chains (S-CH$_3$ for methionine and S-H for cysteine) similar interactions can be envisioned.

Our studies with rtL180C change in the FLLA region leading to resistance to lamivudine are similar to rtL180M-mediated resistance (21). Mutations in the HBV polymerase generated by other nucleosides such as adefovir dipivoxil emphasize the multiple strategies the virus can adopt to elude inhibition (2). Understanding the interaction of the drugs with the active site of the viral enzyme is critical for the development of therapies to combat HBV. Mechanisms by which the virus circumvents the inhibitory activity would shed light on the strategies that need to be implemented for the successful therapeutic intervention. Furthermore, understanding the biology of the resistant virus is critical for the determination of the mechanism of resistance as well as the management of individuals with HBV.

ACKNOWLEDGMENTS

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REFERENCES


