Research Article

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Characterization of novel entecavir resistance mutations

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Background & Aims: Entecavir (ETV) is approved for the treatment of chronic hepatitis B virus (HBV) infections, but the virus can acquire resistance to the drug. This requires lamivudine resistance mutations (LAMr) and at least one additional mutation. Here, we characterized two novel mutations, rtI163V and rtA186T, associated with viral breakthrough (VBT) in an ETV-refractory patient.

Methods: HBV from an ETV-refractory patient was sequenced, and newly identified mutations were inserted into a replication-competent clone by mutagenesis. Clones were analyzed for replication efficacy and susceptibility to ETV *in vitro*. Chimeric mice with human hepatocytes were inoculated with the patient's serum at VBT, and monitored for viral mutation pattern using a next-generation sequencing approach.

Results: RtI163V and rtA186T mutations were detected together with LAMr (rtL180M and rtM204V) at VBT. RtA186T plus LAMr reduced susceptibility to ETV more than 111.1-fold compared with the wild-type clone, while rtI163V plus LAMr resulted in a 20.4-fold reduction. RtA186T significantly reduced viral replication efficacy, while the rtI163V mutation rescued it. Interestingly, the viral mutation pattern in the chimeric mice indicated dominant (or selective) proliferation of a clone containing rtI163V and rtA186T mutations plus LAMr under ETV treatment. Three-dimensional docking simulation indicated that rtA186T reduced the binding affinity of the HBV polymerase to ETV.

Conclusions: VBT in this ETV-refractory patient is attributable to the novel ETV resistance mutations rtI163V and rtA186T. RtA186T was apparently responsible for ETV resistance but the selection of a clone with the double mutation plus LAMr suggests that rtI163V is required to sustain viral fitness.

Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that causes severe diseases such as acute, chronic and fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). More than 240 million people are chronically infected with HBV around the world, and thus at increased risk of developing cirrhosis and HCC. Currently, six drugs are approved for the treatment of chronic hepatitis B including two formulations of interferon, (i.e. standard interferon α-2b (IFN-α-2b) and pegylated interferon α-2a (PegIFN-α-2a)), and five nucleos(t)ide analogues (NAs), lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine [1], and tenofovir for NAs-naïve and drug-resistant patients [1–3].

NAs target the activity of the HBV reverse transcriptase (RT) but a distinct disadvantage of long-term treatment with these drugs is the emergence of drug resistance mutations in the RT region. The rate of drug resistance development has been estimated at around 70% at five years for LAM, 29% at five years for ADV, and 34% at three years for telbivudine [4]. ETV appears less likely to allow development of drug resistance than other NAs, and long-term clinical studies of ETV monotherapy have demonstrated that resistance remains low (around 1.2%) after six years of therapy [5–7]. This property of ETV mitigating against the development of genetic resistance presumably reflects potent viral suppression resulting from efficient and rapid intracellular phosphorylation to generate the active moiety, ETV-triphosphate (ETV-TP), and inhibition of all three activities of the HBV RT (i.e. priming, reverse transcription and elongation of DNA) [8]. However, ETV-resistant variants do emerge, more frequently in patients who harbor variants with key LAM-resistant mutations (rtL180M and rtM204V) than in NA treatment-naïve patients [6,9]. So far, substitutions at the position of rt184, rt202 or rt250 in addition to LAM resistance

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Abbreviations: ETV, Entecavir; LAMr, lamivudine resistance mutations; VBT, viral breakthrough; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; IFN-α-2b, interferon α-2b; PegIFN-α-2a, pegylated interferon; NAs, α-2a nucleos(t)ide analogues; LAM, lamivudine; ADV, adefovir; RT, reverse transcriptase; ETV-TP, ETV-triphosphate; Chimeric Mice Hereafter, chimeric mice with human hepatocytes; HBsAg, Hepatitis B surface antigen; HBcrAg, HBV core-related antigen; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator gene.

substitutions have been shown to confer resistance to ETV; these substitutions often occur in pairs (rtI169T and/or rtM250V, rtT184G, and rtS202I/G) [4,10]. We have previously conducted a cross-sectional study of 49 LAM-refractory patients and 59 naïve patients with chronic hepatitis B, and found that viral breakthrough (VBT) occurred in 26.8% of the LAM-refractory group, but none in the naïve group during weeks 60 to 144 of ETV therapy [9]. Here, we report two novel mutations, rtI163V and rtA186T, in the HBV RT region that were associated with VBT after 54 months of ETV treatment. RtA186T but not rtI163V is the major mutation that conferred ETV resistance in this patient. The viral variants with rtI163V and rtA186T mutations were indeed resistant to ETV treatment, as shown in chimeric mice with human hepatocytes (hereafter referred to as chimeric mice), and rt163V and rtA186T were always found to coexist in the same viral clone, indicating mutual dependency for ETV resistance *in vivo*. Finally, docking simulation suggested that rtA186T, but not rtI163V, reduced the binding affinity of the HBV RT to ETV.

Materials and methods

Patient

A 34-year-old chronic hepatitis B virus-infected patient with severe cirrhosis was started on lamivudine (LAM) monotherapy (100 mg/d) in Hokkaido P.W.F.A.C Sapporo Kosei General Hospital (Fig. 1). Because serum HBV DNA titer remained high after 96 months of treatment, LAM was changed to entecavir (ETV) monotherapy (0.5 mg/d) in September 2003, reducing serum HBV DNA titer by approximately 2.6 log copies/ml after 25 months of ETV treatment. However, a VBT occurred after 54 months and reached 5.5 log copies/ml. After VBT, treatment was changed to adefovir (ADV) (10 mg/d) and LAM (100 mg/d), resulting in a prompt reduction of the HBV DNA titer to below 4 log copies/ml. After 13 months of bitherapy, LAM was switched to ETV (0.5 mg/d) and the HBV DNA titer decreased by more than ten fold, and remained negative thereafter. Written informed consent for ancillary studies was obtained from the patient.

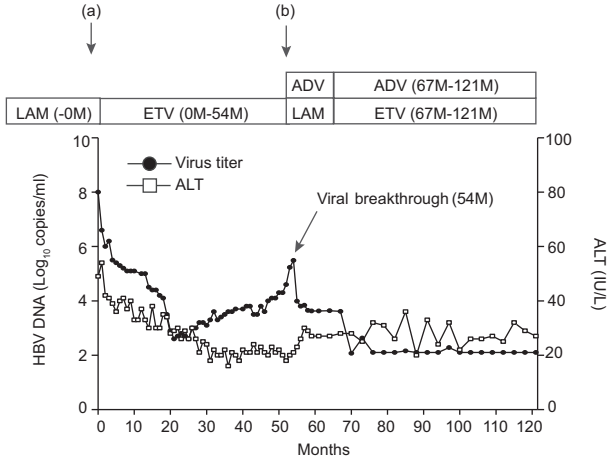

Fig. 1. Clinical course of a patient developing viral breakthrough during ETV therapy. Serum HBV DNA titers (solid circles) and ALT levels (open squares) in a patient with chronic HBV infection at different time points during ETV therapy. Arrows (a) and (b) indicates the time points at which the patient's sera were sequenced for HBV. The duration of each treatment is indicated in the boxes above the graph. The patient was previously treated with LAM (3TC) (100 mg/d), which was later switched to ETV monotherapy (0.5 mg/d). After VBT, ETV was switched to ADV (10 mg/d) and LAM (100 mg/d) biotherapy at 54 months. After 13 months of bitherapy, LAM was replaced by ETV (0.5 mg/d).

Analysis of virological markers

Serum HBV DNA in the patient was measured using Amplicor (lower detection limit: 2.6 log copies/ml) and the TaqMan polymerase chain reaction assay (COBAS TaqMan, Roche Molecular System [lower detection limit: 2.1 log copies/ml]).

HBV DNA titer for chimeric mice was measured by real-time PCR as previously described [11]. Hepatitis B surface antigen (HBsAg) and HBV core-related antigen (HBcrAg) were measured by chemiluminescent enzyme immunoassay using a commercial assay kit (Fujirebio Inc., Tokyo, Japan) as previously described [12,13]. The detection limit of the HBsAg assay and HBcrAg assay is 0.005 IU/ml and 1.0 kU/ml, respectively.

Sequencing and phylogenetic analysis

HBV DNA was isolated from 100 μl of serum collected from the patient before ETV treatment (0 months, arrow (a)) and at the time of VBT (54 months, arrow (b)) (Fig. 1), and sequenced as previously described [14]. Genetic distances were estimated by the six-parameter method, and phylogenetic trees were constructed with the neighbor-joining method, as previously described [15].

Plasmid construction for the in vitro HBV replication model

Table 2 shows the four plasmids used in this study. A plasmid carrying the replication-competent 1.24-fold HBV genotype C2/Ce DNA genome (AB246345) was used as a backbone construct for inserting the different mutations characterized in this study [16]. LAMr mutations (rtL180M and rtM204V) and the newly identified mutations rt163V and rtA186T were inserted into the backbone plasmid by site-directed mutagenesis, using first and second primer sets as described in detail in the Supplementary materials. A 1.24-fold clone based on the C2/Ce strain was digested by *EcoO65*I and *EcoT22*I and ligated with the fragment amplified by fusion PCR carrying the mutations.

In vitro antiviral susceptibility testing of ETV resistance mutants

HuH7 cells were transfected as described previously [12], and in the Supplementary materials. The transfected cells were exposed to titrated concentrations of ETV 5–6 hours later, and then harvested after further 72 hours. Capsid associated HBV DNA was extracted and Southern hybridization performed as previously described [12] with small modifications (see Supplementary materials and methods for the detailed procedure). The IC50 of ETV for each clone was determined as a 50% decrease in the amount of intracellular viral DNA in treated cells relative to untreated cells at the end of the treatment period [12].

In vivo antiviral susceptibility testing of ETV-resistant mutants using chimeric mice

The livers of severe combined immunodeficiency (SCID) mice carrying a transgene expressing urokinase-type plasminogen activator (uPA) gene were replaced by human hepatocytes purchased from BD Biosciences (San Jose, CA) as previously described [17] (hereafter designated chimeric mice). All such mice used in this study were generated by and purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The liver repopulation efficiency was estimated by the human albumin level in the serum, as previously described [17]. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the Ethics Committees of Phoenix Bio Co., Ltd (Permit Number: 1095, 1155). Chimeric mice were housed in specific pathogen–free facilities at the laboratory of Phoenix Bio Co., Ltd. Food and water were provided ad libitum. Chimeric mice were weighed and anesthetized using isofluorane prior to blood collection from the orbital vein.

Groups of three chimeric mice were inoculated with sera obtained from human hepatocyte chimeric mice previously infected with genotype C2/Ce, as described in a previous report [12], or with serum obtained from the patient who had developed VBT 52 months after ETV treatment, as described above. By the time of the first drug administration, serum HBV DNA levels in the C2/Ce-infected chimeric mice ranged between 6.6 and 29.0×10^8 copies/ml, while those in the mice inoculated with the patient's serum ranged between 8.7×10^5 and 1.3×10^6 copies/ml. The mice were then treated with 0.02 mg/kg of ETV (kindly provided by Bristol-Myers Squibb, New York, NY, USA) given orally once a day for 14 days, and followed for another week after ETV withdrawal. Serum HBsAg and HBV DNA levels were monitored every 3–4 days as described above.