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A novel baseline hepatitis B virus sequencing-based strategy for predicting adefovir antiviral response

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ABSTRACT

Adefovir dipivoxil (ADV) is used as first-line monotherapy or rescue therapy in chronic hepatitis B (CHB) patients. In this study, we sought to identify nucleotide changes in the reverse transcriptase (RT) of hepatitis B virus (HBV) at baseline and explore their predictive value for ADV antiviral response. Ultra-deep pyrosequencing (UDPS) was utilized to determine HBV genetic variability within the RT region at baseline and during a 48-week ADV therapy. According to the viral load at the end of ADV treatment, all patients were classified into responders (HBV DNA level reduction of $\geqslant$3 log 10 IU/mL) and suboptimal responders (HBV DNA level reduction of <3 log 10 IU/mL). Based on UDPS data at baseline, we identified 11 nucleotide substitutions whose combination frequency was significantly associated with the antiviral response among 36 CHB patients in the study group. However, the baseline distribution and frequency of rt181 and rt236 substitutions known to confer ADV resistance was a poor predictor for the antiviral response. Compared with baseline serum HBeAg, HBV-DNA and ALT levels, the baseline HBV sequence-based model showed higher predictive accuracy for ADV response. In an independent cohort of 31 validation patients with CHB, the sequence-based model provided greater predictive potency than the HBeAg/HBV-DNA/ALT and the HBeAg/HBV-DNA/ALT/sequence combinations. Taken together, we confirm the presence of ADV resistance variants in treatment-naïve patients and firstly unravel the predictive value of the baseline mutations in the HBV RT region for ADV antiviral response.

1. Introduction

There are about 350 million people chronically infected with hepatitis B virus (HBV) around the world (Ott et al., 2012). Chronic HBV infection has been regarded as a major cause of cirrhosis, liver failure and hepatocellular carcinoma (HCC) in Asia (Liaw, 2009; McMahon, 2009). Treatment of chronic HBV infection has evolved rapidly in recent years, especially after the application of nucleoside/nucleotide analogues (NAs), which directly inhibit the reverse transcriptase (RT) function of HBV DNA polymerase. Several novel NAs, e.g., lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (TBV), and tenofovir (TDF), are currently approved for the management of chronic hepatitis B (CHB) (Dienstag et al., 1999; Lai et al., 2007, 2006; Marcellin et al., 2003, 2008).

Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; AST, aspartic aminotransferase; AUC, area under the curve; BMI, body mass index; CHB, chronic hepatitis B; ETV, entecavir; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; LAM, lamivudine; NPVs, negative predictive values; NAs, nucleoside/nucleotide analogues; ORFs, open reading frames; PCR, polymerase chain reaction; PPVs, positive predictive values; RF, random forest; ROC, receiver operating characteristic; RT, reverse transcriptase; SVM, support vector machine; Sn, Shannon entropy; SDs, standard deviations ; TBV, telbivudine; TDF, tenofovir; UDPS, ultra-deep pyrosequencing.

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The main concern of prolonged antiviral therapy with NAs is the emergence of viral resistance (Zoulim and Locarnini, 2009). HBV resistance generally results in virological and biochemical breakthrough, consequently accelerating liver disease progression. During the development of antiviral resistance, viral populations bearing amino acid substitutions that confer reduced sensitivity to antiviral drugs become predominant (Locarnini et al., 2004; Pawlotsky et al., 2008; Shaw et al., 2006). The majority of resistant HBV variants are thought to emerge during long-term therapy (Zoulim and Locarnini, 2009).

ADV is used as first-line monotherapy or rescue therapy for HBV-infected patients showing failure to LAM treatment, especially in developing countries or areas where novel antiviral drugs are not available (Lee et al., 2013). However, quite a number of patients respond poorly to ADV (Hadziyannis et al., 2003; Lok and McMahon, 2007; Marcellin et al., 2003). Such poor responsiveness is largely affected by host and viral factors (Pallier et al., 2009). Serum HBV markers have been shown to predict antiviral response in patients with CHB (Lee et al., 2011; Rijckborst et al., 2010; Sonneveld et al., 2010; Tseng and Kao, 2013; Yuen et al., 2007; Zhu et al., 2013). However, reliable biomarkers for predicting ADV antiviral therapy have not been established yet.

The genetic heterogeneity of HBV quasispecies has been identified as an important factor in relation to the outcome of antiviral treatment (Chen et al., 2009; Lim et al., 2007; Nishijima et al., 2012; Thai et al., 2012). Although it is generally accepted that the presence of resistant variants at baseline adversely affects antiviral potency, a recent study has shown that the emergence of ADV resistant variants before treatment is not associated with subsequent antiviral response and drug resistance (Rodriguez C et al., 2013). Another study demonstrated that the heterogeneity and complexity of HBV quasispecies is causally linked to the development of drug resistance (Thai et al., 2012). This predisposition can be used to predict the response to antiviral treatment (Thai et al., 2012).

A next-generation sequencing technique, i.e., ultra-deep pyrosequencing (UDPS), is capable of generating vast quantities of data and valuable in assessing virus quasispecies and drug resistance. This technique has been employed to characterize the genetic heterogeneity of HBV quasispecies within or beyond the RT region (Gong et al., 2013; Homs et al., 2012; Ko et al., 2012; Rodriguez-Frias et al., 2013; Rodriguez-Frias et al., 2012; Sede et al., 2012; Solmone et al., 2009). In this study, we examined the HBV genetic variability at baseline and during ADV therapy using the UDPS technique and developed a novel sequencing-based strategy for predicting ADV antiviral response.

2. Patients and methods

2.1. Patients

A total of 36 patients with CHB were enrolled as study group, while another 31 CHB patients were independently selected as validation group. All patients were nucleoside/nucleotide-naïve before receiving 48 weeks of ADV and hepatitis B e antigen (HBeAg)-positive and received at least 48 weeks of ADV (10 mg/-day) treatment between January 2004 and January 2005 at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). Exclusion criteria included the presence of hepatitis C virus (HCV), hepatitis D virus (HDV), or human immunodeficiency virus (HIV) infection, liver cirrhosis, HCC, and alcohol- or drug-induced hepatitis. Written informed consent was obtained from each patient and the protocol was approved by the Ethical Committee of the Chongqing Medical University in accordance with the Declaration of Helsinki (approval number: 2004L01381; 2004L02605; 2005S02624).

Serum virological and biochemical markers were tested for all patients at baseline and 48 weeks after ADV therapy and results were retrieved from patient records. The quality of aliquoted serum was verified by re-checking viral load before UDPS assays performed in 2010. Serum HBV DNA levels were measured using the Roche Amplicor Monitor Test (Roche Diagnostics, Indianapolis, IN, USA). The Architect HBsAg QT immunoassay (Abbott Diagnostic, Wiesbaden, Germany) was used to quantify hepatitis B surface antigen (HBsAg) according to the manufacturer's instructions. Serum HBeAg levels were quantified using a microparticle enzyme immunoassay (AXSYM HBe 2.0; Abbott Laboratories, Abbott Park, IL, USA). HBV genotype was determined with polymerase chain reaction (PCR) amplification and sequencing of the HBV RT region. Serum alanine aminotransferase (ALT) and aspartic aminotransferase (AST) levels were measured using standard enzymatic methods. According to the degree of viral load reduction after 48 weeks of ADV treatment, all patients were classified into two groups: responders, defined as HBV DNA level reduction of $\geqslant 3$ log 10 IU/mL; suboptimal responders, defined as HBV DNA level reduction of <3 log 10 IU/mL (Rodriguez-Frias et al., 2014; Rodriguez C et al., 2013).

2.2. UDPS analysis

Serum HBV DNA at baseline and 48 weeks after ADV treatment was extracted using the QIAamp blood mini kit (Qiagen, Hilden, Germany). Nested-PCR was used to amplify a 261-bp fragment (nt 619–879) including the HBV RT B and C domains, which covers the major NA-resistance-related substitutions. The first round PCR conditions were 1 cycle at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The second round PCR conditions were 1 cycle at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR primers are listed in Supp. Table S1. To minimize misincorporation errors, the high-fidelity DNA polymerase (Primer STAR; Takara, Japan) was used. PCR products were purified with the QIA quick PCR purification kit (Qiagen) and quantified by an Agilent 2100 bioanalyzer (Agilent Life Sciences and Chemical Analysis). DNA adaptors containing primer binding sites for deep sequencing and multiplex identifiers (MIDs) for sample bar coding were ligated into the purified DNA fragments using a GS FLX Titanium Rapid Library Preparation kit (Roche Applied Science). The library was subjected to emulsion PCR for clonal amplification of DNA fragments on water-in-oil emulsion microreactors followed by enrichment and counting of DNA containing beads (GS FLX Titanium LV emPCR kit [Lib-L] and GS FLX Titanium emPCR breaking kit LV/MV 12pc; Roche Applied Science). Subsequently, microbeads were collected and loaded onto the PicoTiter plate of the FLX Genome Sequencer. Finally, sequencing was carried out with the GS FLX method (454 Life Sciences, Roche, Branford, CT) by the company Majorbio Bio-Pharm Technology (Shanghai, China).

2.3. Bioinformatics analysis of UDPS data

UDPS sequencing data were processed as described previously (Cheval et al., 2011). A data filter program written in the Perl script was used for sequence analysis. Filtering criteria included sequence bases with mean quality scores of <13, presence of $\geqslant 10$ low-quality bases or $\geqslant 15$ consecutive identical bases, short length of <281 bp, presence of undetermined bases, and occurrence of a premature stop codon. For complete sequencing of the UDPS fragments of 261 bp in size, a minimal sequence length of 281 bp

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