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Short communication

Quantification of the newly detected lamivudine resistant YSDD variants of Hepatitis B virus using molecular beacons

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Abstract

A real-time based amplification assay with molecular beacons was used to detect and quantify PCR amplicons to discriminate between the newly described Lamivudine-resistant YSDD variant, a known YIDD variant and wild-type Hepatitis B virus (HBV) DNA in the YMDD region of the polymerase gene. Using this assay, we retrospectively analysed samples from two HBV chronically infected Asian twin sisters, starting 9 weeks before therapy, during and between two periods of treatment with Lamivudine. In order to analyse more accurately the dynamics of variant DNA loads during and after therapy, this real time assay was compared to three other mutation analysis techniques, restriction fragment length polymorphism (RFLP), InnoLipa HBV-DR assay and direct sequence analysis. With this technique, new information on the dynamics of variants during and after therapy was obtained.

Keywords: Hepatitis B virus (HBV); HBV DNA monitoring; Lamivudine; Resistance; YMDD motif; Polymerase gene; Molecular beacon

1. Introduction

It has been estimated that more than 300 million individuals worldwide are chronically infected with HBV. Although most infections are acute, this chronic infection may lead to hepatic necroinflammation, fibrosis (Wong et al., 2004), cirrhosis and hepatocellular carcinoma.

Hepatitis B viral load monitoring has become very valuable with the introduction of antiviral therapy like α-Interferon, Lamivudine or Adefovir and is recommended by consensus statements. Lamivudine {(−)2′,3′-dideoxy-3′-thiacytidine} is a chain terminator and inhibits the replication of the HBV virus without influencing the cccDNA replicative intermediate. Escape variants do arise at random and some of these variant strains can replicate. Others and we have found mutations of methionine (amino acid 204 of the reverse transcriptase) in the YMDD motif, located in the C domain of the polymerase gene of HBV (Ling et al., 1996; Niesters et al., 1998). The YVDD and YIDD variants were described before and the YSDD variant has recently been described (Bozdayi et al., 2003; Niesters et al., 2002).

During Lamivudine therapy not only viral load monitoring has become important, but also the early detection of the drug resistant escape variants is necessary. Several techniques have been described for the detection of variants, each with its own advantages and disadvantages (Allen et al., 1999; Pas et al., 2002). However, understanding the dynamics with which these variant viruses do arise during and especially after cessation of therapy is poorly investigated. In this paper, we applied real-time detection with molecular beacons to analyze the dynamics of variant virus asides wild-type virus more accurately. These molecular beacons are stem-loop formed oligonucleotide probes, which open upon hybridization and generate a fluorescence signal (Tyagi and Kramer, 1996). Several different mutation analysis methods were compared for two chronically infected twin sisters who visited our outpatient clinic and we discuss the useful-

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ness of these different techniques in a diagnostic laboratory setting.

2. Materials and methods

2.1. Patient and clinical samples

Two patients of Asian origin, twin sisters, with a chronic Hepatitis B virus infection were treated with 150 mg Lamivudine daily for a period of 753 days. The follow-up period for both patients was until 490 days after the end of the first treatment period, after which both patients started the second period of Lamivudine treatment which we will describe until week 254. Between day 113 and 125, α-interferon was added (7 days with 10 MU and 5 days with 5 MU), but stopped because of side effects. Samples requiring dilution to measure HBV DNA were diluted in known HBV DNA negative serum. All aliquots were stored frozen at −20 °C until use.

2.2. Measurement of HBV DNA in serum

For the accurate measurement of HBV DNA in serum, both the digene hybrid capture (HC II) plate assay, as well as an in-house developed HBV DNA TaqMan assay was used (Niesters et al., 2000; Pas et al., 2000). All assays were calibrated using EUROHEP standards (Heermann et al., 1999).

2.3. Line probe HBV drug resistance assay (InnoLipa-HBV DR)

The InnoLipa HBV-DR assay (Innogenetics, Gent, Belgium) was performed essentially as described before(Stuyver et al., 2000b), using AmpliTaq Gold Taq DNA polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) as enzyme.

2.4. RFLP analysis

In order to develop a more specific screening assay for the YSDD (rtM204S, ATG->AGT) and YIDD/att (rtM204I, ATG->ATT) variants, restriction fragment length polymorphism (RFLP) assays were developed. This RFLP assay detects nucleotide changes in the HBV polymerase using the restriction endonuclease SfcI (CT▼PuPyAG, New England Biolabs, Beverly, MA, USA) for YSDD and SspI (AAT▼ATT, New England Biolabs) for YIDD/att variant. The specific endonuclease site was introduced with a modified sense primer in separate PCR reactions (sense CAC.TGT.YTG.GCT.TTC.AGC.TAT for YSDD and CAC.TGT.YTG.GCT.TTC.AGT.AAT.A for YIDD/att; antisense GTT.YAA.ATG.TAT.ACC.CAA.AG), generating an amplification product of 120 basepairs. Amplicons were restricted for 1 h at 37 °C and the product was run on a 3% Metaphor agarose gel electrophoresis with 0.01% Gelstar as intercalating dye. In the case of a YSDD variant, a fragment of 102 basepairs was detected; if a YIDD/att variant was present a fragment of 99 basepairs was detected. Wild-type HBV DNA was detected using the RFLP assay described before (Allen et al., 1999).

2.5. Sequence analysis

The total HBV genome was sequenced in parts with primers as described before (Stuyver et al., 2000a). These sequence products gave information on both mutations related to Lamivudine resistance, as well as the genotype of the HBV virus. Two microliter amplicon was amplified and precipitated with the DYEnamic™ terminator cycle sequencing kit according to the manufacturer's protocol (Amersham Pharmacia, Roosendaal, The Netherlands). The products were separated on the ABI 373 sequencer (Applied Biosystems) and the sequence data were analysed using Sequence Navigator software sequencer (Applied Biosystems).

2.6. Real time variant analysis using molecular beacons

A real-time amplification system using molecular beacons was developed to detect separate variant strains of HBV specifically for these patients.

Molecular beacons were designed for PCR and tested for specificity by using a thermal denaturation profile. For each molecular beacon three target oligonucleotides (wild type and one of the two variant sequences) were each added to separate tubes containing the TaqMan reaction mixture (see below), in a concentration, which exceeded four times the concentration of the molecular beacon. The third tube did not contain any target. The samples were placed in ABI Prism 7700 Sequence Detection System (Applied Biosystems) and the temperature was decreased from 80 to 25 °C in steps of 1 °C per 30 s. The fluorescence was measured during each step of this thermal denaturation profile.

For wild type, YSDD and YIDD/att variant, a specific quantitative real-time PCR was developed using these molecular beacons. Amplification was performed in a 50 μl reaction mixture, containing 2× TaqMan Universal Master Mix (Applied Biosystems, final $MgCl_2$ concentration 5 mM), 20 pmol of forward primer (5′-AGT.GGT.TCG.TAG.GGC.TTT.CC-3′), 80 pmol of reverse primer (5′-GGG.ACT.CAA.GAT.GTT.GTA.CAG.AC-3′), 20 pmol of specific Molecular Beacon (Table 1) and 10 μl of HBV DNA, isolated using the High Pure Viral Total Nucleic Acid kit (Applied Science, Roche Diagnostics, Almere, The Netherlands). The PCR cycling program was initiated by 2 min at 50 °C and 10 min at 95 °C, after which 45 cycles were run consisting of three steps of 30 s at 95 °C, 30 s annealing step at 50 °C for the YIDD/att beacon or 53 °C for the YMDD and YSDD specific beacon and 30 s extension at 60 °C.

Quantitation of either variant or wild type HBV DNA was performed using separate standard curves of serial diluted