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.

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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 (−1′)-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 besides 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-
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 week 254. Between day 113 and 125, 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).

2.2. 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; AGT→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 (CTG↓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 using a modified sense primer in separate PCR reactions: sense cag.tcg.tyg.gct.ttc.acg.tat for YSDD and cag.tcg.tyg.gct.ttc.acg.tat for YIDD/att. The PCR cycling program was initiated by 2 min at 95°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 all three probe yields was performed using separate standard curves of serial diluted DNA, with the limit of detection 100 genome copies/reaction. A real-time amplification system using molecular beacons was developed to detect separate variant strains of HBV specifically for these patients.

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 microtitre amplicons was amplified and precipitated with the DYEEnam terminator cycle sequencing kit according to the manufacturer’s protocol (Amersham Pharmacia, Rosendaul, The Netherlands). The products were separated on the ABI 373 (Applied Biosystems) and the sequence data were analysed using Sequence Navigator software (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 2X TaqMan Universal Master Mix (Applied Biosystems, final MgCl2 concentration 5 mM), 20 pmol of forward primer (5′-AGT↓AGT-TCG-AGC-TATTTTCC-3′), 80 pmol of reverse primer (5′-GC↓AGC-CAA-ATT-CTG-CTC-3′), 20 pmol of specific Molecular Beacon (Table 1) and 10 μl of HBV DNA, isolated using the High Pure Viral 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 DNA, with the limit of detection 100 genome copies/reaction.
Table 1

The sequences of the molecular beacons used for detection in two amplification systems for specific sites of the HBV polymerase gene are summarised.

<table>
<thead>
<tr>
<th>System</th>
<th>Specificity</th>
<th>Sequence of molecular beacon</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>YMDD</td>
<td>5'-FAM-ctaacctggcttctcagtaaattgagtggttgg-3'</td>
</tr>
<tr>
<td></td>
<td>YSDD</td>
<td>5'-FAM-ctaacctggcttctcagttaaggagtggttgg-3'</td>
</tr>
<tr>
<td></td>
<td>YIDDatt</td>
<td>5'-FAM-ctaacctggcttctcagtaaattgagtggttgg-3'</td>
</tr>
</tbody>
</table>

positive controls of 100% variant or wild type HBV DNA, ranging from 10 million down to 1000 copies/ml in each run. The positive controls were quantified in the quantitative HBV TaqMan assay (Pas et al., 2002). Analysis was performed as described before.

3. Results

3.1. Case report

Patients A and B (twin sisters) were routinely monitored during Lamivudine treatment by HBV quantitative assays. An indication for resistance to Lamivudine was increased HBV DNA serum levels. Screening for any variants was routinely done using InnoLipa HBV-DR assay (see Fig. 1). For sister A no specific band at the rt204 position could be detected in the InnoLipa HBV-DR assay and therefore, additional sequence analysis was performed. The latter method revealed an unknown variant of HBV at the YMDD site in the C region of the polymerase gene of HBV in patient A. The aminoacid methionine at position rt204 was substituted by a serine. This is a double mutation from A TG into AGT. This double mutation also resulted in a change in the reading fame of HBsAg: sW196V. The virus was typed as HBV genotype B (serotype ayw1 according to Norder et al., 1992). Three additional single point mutations were found in the variant strain, one of which was located in the spacer domain of polymerase gene at position sL144F, which did not result in a mutation in preS2. The other two mutations were rtL180M and rtA222T, but only the latter mutation resulted in a sM213I variant in the reading frame of HBsAg. The InnoLipa HBV-DR assay showed a YIDDatt variant in patient B. Therapy was stopped for both patients at week 108.

3.2. RFLP analysis

A specific RFLP assay was developed for the YIDDatt variant of sister B. Although not visible in Fig. 2, variant
HBV DNA (YIDD/att) was visible on gel at week 72 (Table 2) next to wild-type HBV DNA. Wild-type DNA remained detectable until week 85. Within 3 weeks after end of therapy, wild-type virus was detectable again asides YIDD/att variant until end of follow up (week 190).

A RFLP analysis of sister A was described until week 112 before (Niesters et al., 2002). Additional samples after end of therapy (week 126, 139, 161, 172, 178 and 187) were screened and both YSDD and YMDD variants were detected in all samples (data not shown). As described before, we assumed as working hypothesis that an YIDD intermediate with the sequence ATT should precede the occurrence of the YSDD variant. Although we have screened a large number of individual recombinant clones, this intermediate was not found. In addition, we have also performed the YIDD/att specific RFLP on sister B, but also no YIDD/att variant as intermediate was found.

3.3. Thermal denaturation profiles of molecular beacons

In order to improve our understanding of the dynamics of both wild-type and variant viruses during and after Lamivudine therapy, specific molecular beacons were designed to detect and quantify the variant viruses of these two patients. In order to determine the optimal temperature for hybridising to the specific target, thermal denaturation profiles were generated. In Fig. 3, the thermal denaturation profiles for all used PCR beacons are plotted. All beacons hybridise in a specific temperature range to the ideal target. From these profiles the best annealing temperature was selected for both the YMDD and YSDD beacons at 53°C, and for YIDD/att beacon at 50°C.

3.4. Retrospective analysis using molecular beacons

We were able to specifically quantify wild-type (YMDD) and variant (YSDD and YIDD/att) HBV DNA in samples from both sister A and B using molecular beacons. In patient A, HBV viral load declined 4.74 log HBV DNA from 1.30E9 down to 2.32E4 genome equivalents HBV DNA per ml after 30 weeks of Lamivudine therapy. During this period, only wild-type HBV could be detected. At week 76, the YSDD variant was detected asides wild-type HBV in serum, however, not until 9 weeks later (week 85) an increase of more than 1 log total HBV viral load was measured. Viral load of the YSDD variant did rise until end of therapy (week 108) and wild-type viral load was not detectable from week 89 onward. However, within 2 weeks after end of therapy, wild-type virus (YMDD) was detectable at the same HBV DNA level as YSDD variant virus DNA. Viral load of wild-type virus increased and viral load of the YSDD variant virus decreased 3.5 log geq/ml HBV YSDD DNA until start (week 187) of the second Lamivudine therapy, but was still detectable in serum at low levels (7.40E4 geq/ml HBV YSDD DNA). Within 5 weeks (week 192) after start of the second period of Lamivudine therapy, wild-type levels dropped and variant

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Table 2

Presents the time (weeks) at which the YMDD variant in the polymerase gene of HBV was detected in patient A (YSDD) and B (YIDD/att) using the four variant detection assays

<table>
<thead>
<tr>
<th></th>
<th>B InnoLipa HBV-DR assay</th>
<th>RFLP analysis</th>
<th>Sequence analysis</th>
<th>Molecular beacons in PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>No detection*</td>
<td>72</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Patient B</td>
<td>72</td>
<td>72</td>
<td>76</td>
<td>76</td>
</tr>
</tbody>
</table>

* YSDD variant is not specifically detected for in the InnoLipa HBV-DR assay.
YSDD strain levels increased and after 18 weeks (week 205) no wild-type strain was detectable anymore. This remained until end of follow up.

Patient B, the twin sister of patient A, showed similar kinetics pattern for the YIDD/att variant as her sister. The HBV viral load declined with 4.71 log HBV DNA from 1.16E9 down to 2.20E4 genome equivalents HBV DNA per ml after 30 weeks of Lamivudine therapy. During this period, only wild-type HBV could be detected. At week 76, the YIDD/att variant was detected asides wild-type HBV in serum, but the viral load of sister B increased already more than one log at week 61. Viral load of the YIDD/att variant did rise until end of therapy (week 108) and wild-type viral load was not detectable from week 89 onward. However, within 5 weeks after end of therapy, wild-type virus (YMDD) was detectable at the same HBV DNA level as YIDD/att variant virus DNA. This is later than sister A as we had more samples at different time points of sister A available. Viral load of wild-type virus increased and viral load of the YSDD variant virus decreased 1.33 log geq./ml HBV YIDD/att DNA until start (week 187) of the second Lamivudine therapy, but was still detectable in serum (4.70E6 geq./ml HBV YIDD/att DNA). Also in patient B, wild-type levels dropped within 5 weeks (week 192) after start of the second period of Lamivudine therapy and variant YIDD/att strain levels increased. Only, after 67 weeks (week 254) no wild-type strain was detectable anymore. This remained until end of follow up.

4. Discussion

In this paper, we described the viral kinetics of a new, a known variant selected by Lamivudine therapy, and wild-type Hepatitis B virus in two patients who are twin...
HBV DNA measurement combined with sensitive assays to detect Lamivudine resistant strains is the best combination for individual disease management of chronically infected patients. However, new mutations might be detected in the near future that needs constant adaptations of any detection systems.

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