Detection of rtN236T and rtA181V/T Mutations Associated with Resistance to Adefovir Dipivoxil in Samples from Patients with Chronic Hepatitis B Virus Infection by the INNO-LiPA HBV DR Line Probe Assay (Version 2)

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The nucleotide analogue adefovir dipivoxil (ADV) is an effective antiviral treatment for chronic hepatitis B virus (HBV) infection, with resistance to ADV estimated to occur less frequently than resistance to lamivudine treatment. The detection of ADV resistance mutations is necessary during therapy to monitor and anticipate possible treatment failure. The INNO-LiPA HBV DR v2 (LiPA; Innogenetics, Ghent, Belgium) is a DNA hybridization line probe assay for the detection of HBV polymerase mutations associated with resistance to lamivudine and ADV. Evaluation of this assay to detect ADV resistance mutations was performed by analyzing 38 patients treated with ADV. Serial samples taken at 6-month intervals during treatment were available for most patients. A total of 124 samples were analyzed by both LiPA and sequencing. By LiPA analysis, 12 patients (31.5%) were found to have mutations associated with resistance to ADV (rtA181V/T and/or rtN236T). This contrasted with sequence analysis, which found nine patients (24%) with either or both mutations. Twice as many samples were rtN236T positive by LiPA (18 of 124) compared to sequence analysis (9 of 124). LiPA detected the rtN236T mutation at least 6 months earlier than its detection by sequencing in patients for whom consecutive serum samples were available. Although less sensitive, sequencing has the advantage of providing information on other polymerase mutations not represented on LiPA strips. The INNO-LiPA HBV DR v2 assay is a very sensitive and specific assay for the detection of the rtN236T mutation associated with resistance to ADV.

Approval of adefovir dipivoxil (ADV) for the treatment of chronic hepatitis B virus (HBV) infection in Canada was granted in 2003. ADV is a nucleotide analogue that targets the reverse transcriptase activity of the HBV polymerase during viral replication. It has increasingly become a treatment option for HBV infection due to the high rate of lamivudine resistance upon prolonged treatment. However, viral resistance to ADV (ADV-R) has also been shown to develop and increase over time, from 1 to 2% after the first year of treatment to 18% after 4 years of treatment (16). The mutations recognized as causing ADV-R, rtA181V/T and rtN236T (3, 12), are found within the B and D functional domains of the HBV reverse transcriptase, respectively (5). These mutations are independently associated with viral breakthrough, increases in liver enzyme levels, and clinical and biochemical deterioration in liver function (3, 12, 29). Genotypic resistance monitoring is important to anticipate and confirm observed phenotypic resistance so that new treatment options can be instituted.

A new commercial assay kit, the INNO-LiPA HBV DR v2 (LiPA; Innogenetics, Ghent, Belgium), has been developed for the detection of HBV polymerase mutations associated with resistance to lamivudine and ADV. The v2 kit updates the previous INNO-LiPA HBV DR kit by incorporating new oligonucleotide probes for the detection of mutations resulting in ADV-R (rtA181V/T and rtN236T). The kit utilizes a reverse hybridization line probe assay for the detection of wild-type, mutant, or polymorphic codons (rtI80, rtT173, rtI80, rtI181, rt204, and rt236) within the functional domains of the HBV reverse transcriptase. The present study evaluated the LiPA kit for the detection of ADV-R mutations in 38 patients treated with ADV.

MATERIALS AND METHODS

Patient samples. A total of 124 samples from 38 chronic HBV patients undergoing treatment with ADV were collected for analysis. Samples collected at baseline and at 6-month intervals during treatment were available for 31 patients (two to nine samples per patient over a 6-month to 4.5-year period), while only a single time point sample was available for seven patients (ADV therapy time points were from 1.5 to 7 years). Phenotypic and genotypic ADV-R was previously identified in three patients (29) prior to starting the study. Patient samples were submitted to the National Microbiology Laboratory (Winnipeg, Manitoba, Canada) from the Toronto Western Hospital (Toronto, Ontario, Canada) and Hopital Saint-Luc du CHUM (Montreal, Quebec, Canada).

DNA extraction. HBV DNA was extracted from 150 μl of serum by sodium dodecyl sulfate-proteinase K and phenol chloroform extraction methods (21). Extracted DNA pellets were resuspended in 30 μl of sterile distilled water and stored at −20°C until use.

INNO-LiPA HBV DR v2. Extracted DNA was PCR amplified by using the kit-supplied primer mix (sense [5′-CGTGGTGAGCTTCTCCTCAATTTTTC-3′]; antisense [5′-AGAAAAGGCTTGTAAGTTGCGA-3′]) to obtain a biotinylated PCR product specific for a region of the HBV polymerase gene. PCRs included 8 μl of DNA extract, AmpliTaq Gold reaction buffer (Applied Biosystems, Foster City, CA), primer mix, 0.2 mM deoxynucleoside triphosphates (Invitrogen/Life Technologies, Burlington, Ontario, Canada), 2.5 mM MgCl2, and 2 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems) in a total volume of 50 μl. Thermal cycling parameters involved 50 cycles of 94°C for 30 s,
breakthrough and ALT flares. The two patients not showing ADV-R also showed phenotypic resistance in the form of viral (Table 1). Ten of the twelve patients showing genotypic lation between HBV genotype and ADV-R mutation observed.

Sequence analysis software (DNAStar, Inc., Madison, WI).

Sequences were aligned with 10 genotype-specific HBV polymerase coding sequences were translated from each HBV sample nucleotide sequence. There-

sequence for six HBV polymerase codons. Reading of the test sequence analysis and alignments were performed by using Lasergene se-
hybrids. Procedures to prevent contamination during PCR were followed as described previously (23).

A portion of the HBV polymerase gene was amplified by using the primers Spr1A (5'-GTTTCAAGACAGTAAAGCC-3') and antisense (5'-G AAAGGCTTGTGAATGGCG-3'). If necessary, the primers sense (5'-GGT GGACTTCTCTAATTTCATTAG-3') and Spr2A (5'-ACTTTCCAATCAT AAGGCC-3') were used for nested PCR. The Spr1A and Spr2A primers were designed as consensus primers by using an alignment of all HBV genotypes, whereas the sense and antisense primers were similar to the LiPA kit primers. Thermal cycling parameters involved 35 cycles of 94°C for 30 s, 56°C for 30 s (first stage) or 50°C for 30 s (second stage), and 72°C for 40 s. Reaction tubes for PCR were prepared as described above and contained 5 µl of DNA extract or 2 µl of the first-stage PCR product.

If the first-stage product (1,049 bp) was visible after agarose gel electrophore-
sis, it was gel purified prior to cycle sequencing with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using BigDye v3.1 terminator chemistry. If no product was visible after PCR, a nested PCR was carried out to increase the sensitivity of detection and to provide an amplicon (730 bp) for sequence analysis. Sequence data obtained were used to determine the genotype of each viral sample using the NCBI genotyping tool (24). Polymerase protein coding sequences were translated from each HBV sample nucleotide sequence. There-

HBV genotypes. All HBV genotypes, other than genotype F, were represented in the 124 serum samples analyzed. Geno-
type A was the most predominant (11 [of 38 patients]), fol-

RESULTS

HBV genotypes. All HBV genotypes, other than genotype F, were represented in the 124 serum samples analyzed. Geno-
type A was the most predominant (11 [of 38 patients]), fol-

Detection of ADV-R mutations by LiPA. The LiPA strip contains 34 reaction lines, including 2 control lines and 32 HBV probe lines representing wild-type, mutant, or mixed sequence for six HBV polymerase codons. Reading of the test strips was relatively simple; however, the sheer number of probe lines on each strip, the presence of faint bands, or the absence of bands (see below) at certain codon sites sometimes made interpretation of test strips difficult.

Table 1 shows the results of LiPA testing of the 38 patients. Dual mutations (rtA181V/T and rtN236T) were observed in six (16%) patients, while six other patients had either rtA181V/T alone (n = 3) or rtN236T alone (n = 3). There was no corre-

phenotypic resistance both had the rtA181V/T mutation alone; one patient demonstrated no phenotypic resistance, while the other patient was observed to have a slow response to ADV (defined as HBV DNA levels remaining at greater than 100,000 copies/ml after 1 year of treatment).

The rtN236T mutation developed in nine patients, including seven who were previously treated with lamivudine and had confirmed phenotypic and genotypic resistance to lamivudine prior to starting ADV therapy. LiPA analysis demonstrated that the rtN236T mutation and the rtM204V/I/S mutation (i.e., the major mutation associated with resistance to lamivudine) (5) appear to be mutually exclusive and develop sequentially after a switch from lamivudine therapy to ADV. A mutual exclusivity between these two mutations was observed in 13 of 16 samples from the seven lamivudine-resistant patients having LiPA-identified rtN236T. The observed sequential develop-

Indeterminate results. Several samples (6 of 124) were nonamplifiable; 2 samples could not be amplified by LiPA or sequence primers, while 3 samples were nonamplifiable by LiPA primers alone and 1 sample was nonamplifiable by se-

Concordance between LiPA and sequencing. The rtN236T mutation was detected by LiPA in twice as many samples as was detected by sequencing (18 of 124 and 9 of 124, respectively), whereas LiPA detected the rtA181V/T mutation in 22 of 124 samples compared to 17 of 124 samples detected by sequencing. Table 2 presents the concordance results between LiPA and sequencing for the 117 samples amplifiable and
compared by both methods. After LiPA analysis, one sample did not hybridize with any of the codon 236 oligonucleotides present on the strip (the sample was determined to be wild type by sequence analysis); thus, concordance analysis at codon 236 was carried out with the 116 remaining samples that could be directly compared. Complete concordance between LiPA and sequencing was observed with 110 of 117 (94%) samples at codon 181 and with 105 of 116 (90.5%) samples at codon 236 (Table 2).

Discrepant results were observed for 7 of 117 (6%) samples at codon 181 and with 11 of 116 (9.5%) samples at codon 236. For the most part, discrepant results were due to the mutations being detectable by LiPA but not by sequence analysis. A single time point sample from one patient was nonamplifiable by LiPA but had both rtA181V and rtN236T by sequence analysis. For patients for whom 6-month follow-up samples were available after detection of ADV-R, sequencing and LiPA results became concordant in all cases, except for two patients (one patient had rtA181V/T at two consecutive time points, as detected by LiPA but not sequencing, and one patient had rtN236T at three consecutive time points, as detected by LiPA but not sequencing). LiPA and sequence analysis of mutant/wild-type amplified mixtures having an increasing ratio of mutant to wild-type sequence demonstrated the increased sensitivity of LiPA compared to direct sequencing (data not shown).

Detection of other mutations associated with ADV-R as detected by sequencing. ADV-R has been associated with further mutations within the A and D domains and the C-D interdomain of the reverse transcriptase region of the HBV polymerase (4). These mutations include rtV84M, rtS85A, rtV214A, rtQ215S, rtP237H, and rtN238T/D. Mutations or polymorphic changes at these sites were observed by sequence analysis in 10 patients (Table 3). None of the patients with these other mutations alone demonstrated clinical or phenotypic ADV-R (follow-up ranged from 6 months to 1.5 years).

**TABLE 2. Concordance between LiPA and sequence analysis**

<table>
<thead>
<tr>
<th>Codon</th>
<th>Strain type determined by sequence analysis</th>
<th>No. (%) of samples determined by INNO-LiPA to be:</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt181</td>
<td>Mutant</td>
<td>16 (13.7)</td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>6 (5.1)</td>
</tr>
<tr>
<td>rt236</td>
<td>Mutant</td>
<td>8 (6.9)</td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>10 (8.6)</td>
</tr>
</tbody>
</table>

a Concordance between LiPA and sequence analysis for 117 comparable PCR-positive samples.

b Concordance between LiPA and sequence analysis for 116 comparable PCR-positive samples.

to detect specific HBV nucleotide variations in a more sensitive manner compared to sequencing. Based on 6-month follow-up specimens from individuals on ADV therapy, LiPA detected resistance mutations within 6 months prior to their detection by sequence analysis. Minority quasispecies can be detected by sequencing after cloning and sequencing of individual clones of a PCR product; otherwise, sequence analysis has a relatively low limit of sensitivity (approximately 25%) for the detection of low-level variants (3, 20, 21). LiPA allows the detection of mixtures of both wild type and ADV-R mutants within individual samples that may not be detected by sequence analysis. Thus, LiPA detects minority quasispecies in a more sensitive manner, prior to their expansion toward a dominant species.

Much data has accumulated to support the correlation between the HBV genotype and the clinical response to antiviral therapy, and yet there are still conflicting reports (1, 11, 14). Genotypes A and B appear to respond better to IFN-α therapy than genotypes D and C (8, 30), whereas genotypes A and B develop lamivudine resistance more rapidly or more frequently than genotypes D and B (2, 32). In contrast, several reports have found no association between genotype and the development of lamivudine resistance mutations (19, 27). A lack of correlation between HBV genotype and the development of ADV-R was observed in the present study, although a larger study is required to better understand the relationship. A recent report suggested that HBV genotype D is associated with an increased risk of ADV-R; however, very few ADV-R patients (n = 5) were included in the present study (9).

One disadvantage of the LiPA kit is the limited scope of mutations represented on the assay strip, based on current knowledge of ADV-R and lamivudine resistance mutations. Thus, the inclusion of newly recognized mutations associated with antiviral resistance will require the development of new assay strips. This problem will likely be addressed in the future by changing the assay format to a microarray chip, thus allowing detection of multiple genomic features. Aside from antiviral selection pressure, the lack of proofreading activity by the HBV polymerase, coupled with the rapid replication rate of the virus ensures that polymorphisms or mutations occur frequently. The mutations known to result in ADV-R are rtN236T (3) and rtA181V/T (12). However, newly identified mutations within the A and D domains and C-D interdomain of the HBV reverse transcriptase have also been associated with ADV-R (4, 5). These mutations, including rtV84M, rtS85A, rtV214A, rtQ215S, rtP237H, and rtN238T/D, are known to cause a decrease in sensitivity to ADV in vitro (4). Several of these specific mutations, as well as polymorphic

**TABLE 3. Detection of other mutations within the HBV polymerase**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amino acid change (no. of patients)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V84</td>
</tr>
<tr>
<td>A</td>
<td>M (1)</td>
</tr>
<tr>
<td>C</td>
<td>H (1)</td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>P (1)</td>
</tr>
</tbody>
</table>

* Amino acid changes are indicated by single-letter coding: methionine (M), proline (P), histidine (H), threonine (T), and alanine (A). The number of patients with the indicated amino acid change is given in parentheses.

**DISCUSSION**

Genotypic resistance to various approved (3, 15, 26, 28) or experimental (10, 26) nucleos(t)ide analogue treatments for chronic HBV often leads to treatment failure. Thus, early detection of these mutations is necessary to understand clinical changes and expedite treatment modification. The LiPA kit allows the detection of mutations resulting in ADV-R in chronic HBV patients. Consistent with other reports (13, 18, 22), LiPA was found
changes at these sites, were observed in approximately one-quarter of the patients studied. There was no correlation observed between clinical resistance and the presence of these other mutations; therefore, their association with ADV-R in vivo requires further investigation. Interestingly, most of the other mutations were first observed in patients prior to ADV treatment, suggesting that lamivudine therapy selected for these ADV-R mutations. Lamivudine and ADV each possess a different mechanism of action (5, 25, 31) and, to date, do not share cross-resistance (17). Nucleos(t)ide analogue combination therapy with non-cross-resistant analogues has been recommended in order to avoid such preselection of resistance during monotherapy (7, 33).

Overall, the LiPA assay performed well and, compared to sequencing, was able to sensitively and specifically detect the mutations rtA181V/T and rtN236T that result in ADV-R. The LiPA assay involves a single-stage PCR and reverse hybridization protocol very similar to LiPA assays currently on the market (6, 18, 22), although the HBV DR v2 assay contains the most probe lines per strip of the LiPA-based HBV assays available. LiPA has also been designed for full automation of hybridization, washing, and color development steps (Auto-LiPA). Thus, the standardization of assay procedures coupled with the use of scanning software to provide an objective reading and interpretation of strip test results should assist in data collection and prevent possible indeterminate or negative results due to operator error.

REFERENCES