Hepatitis B Virus Containing the I233V Mutation in the Polymerase Reverse-Transcriptase Domain Remains Sensitive to Inhibition by Adefovir

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An isoleucine-to-valine change at position 233 (rtI233V) of hepatitis B virus (HBV) polymerase was recently reported to cause decreased in vitro susceptibility to, and treatment failure of, adefovir dipivoxil (ADV). To further evaluate these findings, we screened our ADV clinical-study sequence database of 853 patients and identified 4 who, at baseline, had HBV with this mutation. All 4 patients responded to treatment with ADV, with a median change in HBV DNA levels of 4.0 log10 copies/mL after 48 weeks of treatment. Phenotypic evaluation of clinical isolates and of a laboratory strain with the rtI233V mutation demonstrated their full susceptibility to adefovir in vitro, and HBV with the rtI233V mutation developed in none of the patients.

Adefovir dipivoxil (ADV) is a nucleotide analogue that is used for the treatment of patients with chronic hepatitis B, including patients infected with HBeAg+ or HBeAg− types of hepatitis B virus (HBV), lamivudine-resistant HBV, liver transplant, and HIV coinfection [1]. On the basis of results on ~1000 patients enrolled in clinical studies of ADV, 2 mutations associated with resistance to adefovir were identified in the polymerase region of HBV: rtN236T and rtA181V [2]. These 2 mutations were demonstrated to have a strong correlation with clinical-treatment failure and to confer a statistically significant reduction of drug susceptibility in vitro [3]. The cumulative probability of mutations associated with resistance to adefovir is 0%, 3%, 11%, 18%, and 29% after 1, 2, 3, 4, and 5 years, respectively [4]. An isoleucine-to-valine change at position 233 (rtI233V) of HBV polymerase was recently reported to cause both primary resistance to ADV in 3 patients, as evidenced by a lack of suppression of HBV DNA levels on initiation of treatment with ADV, and a decrease in susceptibility to adefovir in vitro [1]. The aim of the present study was to evaluate the incidence of the rtI233V mutation and to evaluate its impact on treatment with ADV in a large cohort of patients enrolled in clinical studies of ADV.

**Patient and methods.** Patients from 4 clinical studies of ADV were included in this evaluation: studies GS-98-437 and GS-98-438 were placebo-controlled phase 3 randomized trials designed to evaluate the efficacy of 10 mg of ADV over a 96-week period, in patients with HBeAg+ and HBeAg− chronic hepatitis B [5, 6], respectively; study GS-98-435 was an open-label phase 3 compassionate-use study designed to evaluate the safety and efficacy of 10 mg treatment with ADV in patients with lamivudine-resistant chronic hepatitis B either before or after liver transplantation [7]; and study GS-98-412 (extension phase) was a phase 2 dose range–finding study of ADV. All studies were approved by local Institutional Review Boards, and all of the patients in these studies provided written informed consent.

Levels of HBV DNA in the serum were determined by the Roche Amplicor polymerase chain reaction (PCR) assay, with 1000 copies/mL as the lower limit of quantitation. Genotypic analysis of the polymerase reverse-transcriptase (RT) domain (rt1–rt344) of HBV was performed by standard dideoxy sequencing techniques, as described elsewhere [8]. HBV DNA was extracted from patients’ sera and was PCR-amplified by use of primers designed to amplify the entire virus genome. Genome-length PCR products were ligated into the pHY106 vector [9], and the ligation mixture was transformed into competent cells for amplification. This process results in a cloned HBV quasi-species pool representing the predominant viral population in patients’ sera, which was confirmed through sequencing of the polymerase RT domain [10]. Phenotypic evaluations of the HBV DNA pools were performed in HepG2 cells by Southern blot analysis of the intracellular HBV replicative intermediates [11]. Regression analyses of antiviral data (based on the measurement of double-stranded and single-stranded HBV DNA replicative intermediates) were performed by use of TableCurve 2D software, and best-fit equations were used to calculate the EC50 values. Assays were performed a minimum of 3 times, and EC50 values were reported as an average of all tests.
with the pHY92 wild-type laboratory strain of HBV serving as a control.

To evaluate the effect of HBV with the rtI233V mutation in the absence of patients’ background polymorphisms, the rtI233V mutation was generated in a wild-type genotype D laboratory strain, pCMVHBV. The mutation was introduced by the Quick Change Site Directed Mutagenesis Kit (Stratagene). The presence of the rtI233V mutation was confirmed through sequencing. Susceptibility testing was performed in HepG2 cells, in the manner described above for clinical isolates. The interassay variability of susceptibility as determined by the Southern blot assays was \( \leq 2\)-fold of the mean values.

**Results.** A review of the HBV polymerase genotype results obtained from 853 patients before treatment with ADV—that is, at baseline—identified 4 patients (0.5%) as having HBV with the rtI233V mutation. Patient A was a 65-year-old white male with HBeAg\(^{-}\) HBV (genotype D, study GS-98-438) who received 10 mg of ADV for 5 years. At baseline, this patient had HBV with a mixture of 233I/V without any other conserved site mutations in the RT domain of polymerase. He quickly responded to treatment with ADV; the levels of HBV DNA in his serum fell to \(<1000\) copies/mL by week 8 and remained there throughout his 5 years of treatment with ADV therapy (figure 1A). Patient B was a 51-year-old white male with the HBeAg\(^{-}\) type of HBV (genotype D, study GS-98-435) who received 10 mg of ADV for 3 years. This patient had previously been treated with lamivudine for 3 years and had developed lamivudine-resistant HBV (rtL180M/M204V) and liver decompensation. After 48 weeks of treatment with ADV, the levels of HBV DNA in the patient’s serum decreased by 4.2 log\(_{10}\) copies/mL (figure 1A). Continued treatment with 10 mg of ADV resulted in undetectable HBV DNA by year 3. Patient C, a 25-year-old Asian male (genotype C), and patient D, a 34-year-old Asian female (genotype C), had chronic hepatitis B with HBeAg\(^{+}\) HBV (study GS-98-437). Patient C was randomized to receive 30 mg of ADV, and patient D was randomized to receive 10 mg of ADV. No other conserved
site mutations in the polymerase RT domain of HBV were observed in these patients. After 48 weeks of treatment, the level of HBV DNA levels in patient C serum decreased by 4.7 log10 and was <1000 copies/mL (figure 1B). Patient D also had a response to ADV at 48 weeks; the level of HBV DNA in her serum decreased by 2.2 log10 copies/mL (figure 1B). Because of a misallocation of the study drug that resulted in the patients receiving alternating doses of placebo or ADV during year 2 of the study, both patient C and patient D had intermittent increases in the level of HBV DNA in their sera. On the reinitiation of treatment with 10 mg of ADV, the level of HBV DNA in the serum of patient C again was <1000 copies/mL, and the level of HBV DNA in the serum of patient D had a 2.9-log10 decrease.

All of the patients for whom baseline genotypes were available were followed for up to 5 years of treatment with ADV, and genotypic analysis was performed on all patients with either detectable levels of HBV DNA (on or after week 48) or HBV-DNA rebound (i.e., 1 log10 increase, from nadir in level of HBV DNA). Patient D also had a response to treatment with ADV in a manner similar to that observed in the overall clinical studies of ADV [1], regardless of HBeAg status, HBV genotype, or the presence of concurrent rtL180M/M204V mutations. None of these 4 patients experienced a confirmed rebound in HBV DNA while being treated with ADV, and in 3 of them the levels of HBV DNA decreased to below the assay’s limit of quantitation. These clinical data were supported by an in vitro phenotypic evaluation of isolates from the patients as well as of a laboratory virus with the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>Adefovir EC50, μmol/L</th>
<th>Average change from wild-type EC50*, n-fold</th>
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<tr>
<td>PHY92</td>
<td>Wild type</td>
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<td></td>
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<tr>
<td>Patient A</td>
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<td>ND</td>
</tr>
<tr>
<td>Patient B pool</td>
<td>I233V</td>
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<tr>
<td>Patient D pool</td>
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<tr>
<td>pCMV HBV</td>
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<tr>
<td>pCMV HBV+ I233V</td>
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<td>0.87±0.34</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**NOTE.** ND, not determined (i.e., unable to amplify levels of HBV DNA serum sufficiently to allow phenotypic evaluation).

* Calculated as the average ratio of mutants’ EC50 to wild-type’s EC50.

Discussion. Because of the high estimated error rate of the polymerase RT domain of HBV (10^-4/base/replication cycle) and the typically high levels of HBV production (~10^11 virions/day) in patients infected with chronic hepatitis B, it is not surprising that an HBV variant containing the rtI233V mutation could occur. Our in vitro phenotypic data indicated that the replication efficiencies of the variants containing rtI233V were similar to that of a wild-type laboratory strain, a result that is in agreement with the facts that (1) the variants containing rtI233V existed at baseline and (2) rtI233V is a natural polymorphism. The frequency of this HBV variant in the patients, 4/853 (0.5%), was low and is similar to the 3/500 (0.6%) previously reported by Schildgen et al. [12] on the basis of a search of sequences available in GenBank, although the clinical outcomes observed in them differ from the results described by Schildgen et al. [12]. In the present study, the 4 patients with HBV with the rtI233V mutation responded to treatment with ADV in a manner similar to that observed in the overall clinical studies of ADV [1], regardless of HBeAg status, HBV genotype, or the presence of concurrent rtL180M/M204V mutations. None of these 4 patients experienced a confirmed rebound in HBV DNA while being treated with ADV, and in 3 of them the levels of HBV DNA decreased to below the assay’s limit of quantitation. These clinical data were supported by an in vitro phenotypic evaluation of isolates from the patients as well as of a laboratory virus with the
rtI233V mutation, all of which remained fully sensitive to inhibition by adefovir.

Several differences between the present study and that described by Schildgen et al. could account for the differences in patients’ outcomes. In the study by Schildgen et al., all 3 patients who had previously been treated with lamivudine, whereas in our study, 3 of the 4 patients who, at baseline, had HBV with the rtI233V mutation had not been treated with lamivudine. The course of ADV treatment also was different between the 2 groups. For 1 of the patients in the study by Schildgen et al., ADV was substituted for tenofovir disoproxil fumarate (TDF), with a subsequent increase in HBV DNA. Tenofovir and adefovir have similar anti-HBV activity in vitro [13]; however, whereas a standard dose of ADV is 10 mg, a standard dose of TDF is 300 mg, which represents a 30-fold increase in drug. Data from other studies have demonstrated that 300 mg of TDF has greater antiviral activity than does 10 mg of ADV [14] and that in patients with HBV in whom TDF is replaced by ADV, the levels of HBV DNA in the serum increase [15]. In the remaining 2 patients, the observed effect of treatment with ADV was based on 1 unconfirmed determination of HBV DNA. In the present study, all of the patients with HBV with the rtI233V mutation were followed for >48 weeks of treatment, thereby allowing for confirmation of the response to ADV as well as for evaluation of subsequent loss of antiviral activity. None of these patients experienced a confirmed rebound in levels of HBV DNA on treatment with ADV. Phenotypic evaluation of HBV quasi-species pools obtained from patients with HBV with the rtI233V mutation and of a laboratory strain with this mutation demonstrated sensitivity to inhibition by adefovir, a finding that is in agreement with the clinical response observed in these patients. By contrast, phenotypic evaluation of HBV obtained from patients who developed the ADV resistance–associated rtN236T mutation demonstrated up to a 14-fold reduced susceptibility to ADV in vitro [1]. Finally, HBV with the rtI233V mutation developed in no patient in any of our ADV clinical studies.

Nonviral factors such as drug adherence, differences in drug metabolism, and natural fluctuations of HBV-DNA necessitate the need for frequent monitoring of levels of HBV DNA in patients being treated, to adequately assess efficacy. The differences between our data and those previously reported are indicative of the current lack of standardization regarding resistance to HBV; there are no HBV-DNA assays approved by the Food and Drug Administration, and the use of nonvalidated assays originally developed for research purposes is not uncommon. Likewise, there are no widely used, validated in vitro phenotypic assays. This lack of standardization places a burden on physicians and scientists to carefully define and validate, at the clinical and scientific level, drug-resistant HBV. In conclusion, the present clinical results, along with the associated in vitro phenotypic results, demonstrate that HBV containing the rtI233V mutation in the polymerase RT domain remains sensitive to ADV treatment and that it is not associated with resistance to adefovir.

References

3. Qi X, Xiong S, Yang H, Miller MD, Delaney W IV. In vitro susceptibility of HBV polymerase encoding mutations acquired during adefovir dipivoxil therapy to other anti-HBV agents [oral 171]. In: 57th annual meeting of the American Association for the Study of Liver Diseases (Boston), 2006