Background: HBV variants rtA181V/T, rtN236T and rtI233V, which confer resistance to adefovir dipivoxil (ADV), are not detected in many non-responding patients. Virological characteristics useful for predicting response have not been clearly elucidated. We determined pretreatment virological markers to predict non-response and possible emergence of new variants during therapy.

Methods: This longitudinal study included 41 patients with chronic hepatitis B virus (HBV) infection receiving ADV monotherapy or ADV plus lamivudine (3TC). A fragment of HBV polymerase including catalytic domains was analysed for ADV-resistant variants.

Results: Complete virological response (CVR; HBV DNA <2.5 log_{10} copies/ml) was observed in 15 (36.6%) patients and partial virological response (PVR; HBV DNA <4 log_{10} copies/ml) in 23 (56.1%) patients. On multivariate analyses, hepatitis B e antigen (HBeAg) status was independently associated with CVR (hazard ratio [HR]=0.27, P=0.002) and PVR (HR=0.21, P<0.001) and viral genotype with CVR (HR=0.13, P=0.01). Predictive values for HBeAg were 88% for PVR in HBeAg-negative and 79% for non-CVR in HBeAg-positive patients. Predictive values for viral genotype were 93% for non-CVR and 72% for non-PVR for genotype A. On sequencing, variant rt217R (associated with subgenotype A2) was predictive of non-CVR (100%) and non-PVR (72.7%); the rtS219A variant emerged during therapy in three non-PVR patients. Both positions are located in a region likely to be related to the substrate union site, as predicted by our structural model of the HBV polymerase.

Conclusions: Virological pretreatment characteristics (HBeAg, viral genotype and rtL217R polymorphism) are potentially associated with ADV response. HBV polymerase structural modelling has provided a hypothesis to explain the molecular mechanism for ADV resistance associated with rtR217.

Introduction

Adefovir dipivoxil (ADV) is active against wild-type and lamivudine (3TC)-resistant hepatitis B virus (HBV) strains. ADV resistance has been associated with the selection of the rtA181V/T and rtN236T variants and with baseline presence of rtL233V [1–3]. ADV resistance is less frequent than 3TC resistance and occurs relatively late [4]. No cases were documented during 48 weeks of administration in chronic hepatitis B (CHB) patients who were positive for hepatitis B e antigen (HBeAg) or in HBeAg-negative patients [5]. A recent analysis showed resistance rates of approximately 3% at 2 years of treatment, 11% at 3 years, 18% at 4 years and 29% at 5 years [6]. The low rates of resistance to ADV could be related to a high degree of structural similarity between the ADV molecule and the native substrate, dATP, thus limiting steric discrimination by the viral polymerase [7]. A significant number of patients who do not show selection of HBV mutants are non-responders to ADV therapy, suggesting that treatment failure could be caused by other factors, such as the presence of a primary HBV-resistant variant, host-related factors or even ADV underdosing [8].

In the design of CHB therapy, considerable effort has been directed toward identifying factors predictive...
of response for use before establishing treatment. The hepatitis B genotype is known to affect the response to interferon therapy, with genotypes A and B showing higher response rates than the other genotypes [9]. In 3TC treatment, specific viral sequences and certain clinical profiles, such as low initial HBV DNA levels and moderate or high alanine aminotransferase (ALT) levels, have correlated to failure or to long-term response [10]. Moreover, HBV adw subtype (genotype A) has been associated with a 20-fold higher risk of developing 3TC resistance [11]. A recent report has shown that genotype D is associated with a 15-fold higher probability of HBeAg loss in ADV therapy [12].

In this study we performed a longitudinal analysis of the HBV polymerase sequence in serum samples of patients with CHB obtained before and during ADV therapy to identify specific pretreatment virological markers potentially linked to treatment failure and to analyse the emergence of HBV polymerase variants that might be associated with antiviral escape.

Methods

Patients

This retrospective study included CHB patients receiving ADV as monotherapy or in combination with 3TC, attending Vall d’Hebron University Hospital (Barcelona, Spain) before January 2007. Patients were excluded if they had decompensated liver cirrhosis, hepatocellular carcinoma, coinfections (HIV, hepatitis C virus and hepatitis D virus) or other concomitant liver diseases, had received a liver transplant or were taking immunosuppressive medication. Patients under treatment for <12 months and those with missing data for clinical, biochemical, serological or virological results at baseline or at every 3 months during treatment were also excluded.

The study population consisted of 41 CHB patients treated with 10 mg/day of ADV as monotherapy (30 patients) or in combination with 100 mg/day of 3TC (11 patients). Of the 41 patients, 34 had documented genotypic 3TC resistance and 7 patients were treatment-naive. A total of 24 (58.5%) patients were HBeAg-positive. Serum samples were collected at baseline and at every 3 months during treatment in all patients. Median treatment duration was 24 months (range 12–60). Liver biopsy was performed in all patients. The choice of monotherapy or combination therapy was made at the discretion of the attending physician. Initially, all patients were switched to ADV, whereas in the past few years there has been a tendency to use combination therapy. Written informed consent for participation was obtained from all patients and the study was approved by the Ethics Committee of Vall d’Hebron University Hospital.

To determine predictive factors associated with response or non-response to ADV therapy, patients were classified by serum HBV DNA levels at the end of treatment follow-up according to two criteria: complete virological response (CVR) when serum HBV DNA was undetectable by quantitative real-time PCR with a sensitivity of <5 × 10^2 copies/ml (approximately <2.5 log_{10} copies/ml) and partial virological response (PVR) when HBV DNA was <10^4 copies/ml (<4 log_{10} copies/ml). PVR included patients with a suboptimal response and HBV DNA levels in the range of those observed in inactive HBV carriers [13,14]. Statistical calculations were performed with both criteria. At the end of follow-up, patients with HBV DNA<5 × 10^2 copies/ml were classified as CVR (as well as PVR), patients with HBV DNA>5 × 10^2 and <10^4 copies/ml as non-CVR (but also PVR) and patients with HBV DNA>10^4 copies/ml as non-PVR. In addition, these last patients categorized to non-PVR were, by definition, also non-CVR and therefore total non-responders. For both the CVR and PVR criteria, normal ALT levels were required (<35 IU/l).

Serological testing

Hepatitis B surface antigen (HBsAg), HBeAg and antibody to HBeAg (anti-HBe) were determined by Enzyme immunosassay (Vitros® HBsAg, HBeAg and anti-HBe, respectively; Ortho Clinical Diagnostics, Inc., Bucks, UK).

HBV DNA quantitation, HBV polymerase sequencing and genotyping

HBV copy number was quantified with a real-time PCR as previously described [15]. The detection limit of the PCR assay was 5 × 10^3 HBV DNA copies/ml. Direct sequencing of the HBV polymerase was used to investigate viral resistance-associated mutations, amino acid changes related with ADV treatment and HBV genotype as previously described [16].

Characterization of subgenotypes A1 and A2 was performed by amino acid analysis at position 129 of the HBV reverse transcriptase (leucine for A1 and methionine for A2) [17] and amino acid positions 207 and 209 of the HBV surface protein (asparagine/leucine for A1 and serine/valine for A2) [18]. A total of 185 complete HBV sequences from the GenBank were used to establish consensus sequences for each HBV genotype.

HBV polymerase structural modelling

The basic local alignment search tool (BLAST) program [19] was used to search for the best template in the Protein Data Bank sequence database for homology modelling [20]; the query sequence was the HBV sequence with the GenBank accession number X02763. We began with BLAST alignment, which is automatic and based only on sequence conservation. Then the alignment was refined according to functional knowledge of the two proteins.

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involved in order to preserve alignment of residues known to be functionally important in both sequences. The Modeller 8 version 2 software was used to build several models [21], which were then checked according to structural criteria to identify similar structures and energy criteria to finally identify the best model.

**Statistical analyses**

Time-to-event analyses was carried out by Kaplan–Meier cumulative incidence curves compared using log-rank tests, as well as univariate and multivariate Cox proportional hazard models of relevant prognostic variables. Covariates included in the univariate and the multivariate Cox models were either binomial (gender, treatment group, liver histology, baseline HBV genotype, rtL217R and HBeAg status) or continuous variables (age, baseline serum HBV DNA and ALT levels). All binomial and continuous variables were included together in the same multivariate Cox model. The hazard ratio (HR) or odds ratio were presented with 95% confidence intervals (CI) and the Mann–Whitney U test was used to compare quantitative variables and the Mann–Whitney U test was used to compare qualitative variables and the Mann–Whitney U test was used to compare quantitative variables. All statistical tests were two-sided and significance was set at a P-value of <0.05. The SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analyses.

**Results**

Among the 41 CHB patients, 14 (34%) were genotype A (3 subgenotype A1 and 11 subgenotype A2), 25 (61%) genotype D and 2 (5%) genotype F. At baseline, median serum HBV DNA and ALT levels were 7.1 log<sub>10</sub> copies/ml (range 5.1–8.8) and 72 IU/l (range 50–720), respectively. Baseline demographic, virological, serological and histological characteristics of the study population are shown in Table 1.

**Virological response to ADV therapy and the predictive value of HBV genotype and HBeAg status**

According to the previously defined criteria of response, 15 (36.6%) patients achieved CVR (HBV DNA<2.5 log<sub>10</sub> copies/ml or partial virological response; PVR; end follow-up HBV DNA<4 log<sub>10</sub> copies/ml) and variants, rtR217 and rtA219, grouped by HBV genotype and hepatitis B e antigen (HBeAg) status. *Mean (range). ADV, adefovir dipivoxil; CAH, chronic active hepatitis; LC, liver cirrhosis; 3TC, lamivudine; +, positive; -, negative.

**Table 1. Baseline characteristics of the study population**

<table>
<thead>
<tr>
<th>HBV genotype</th>
<th>HBeAg status</th>
<th>Treatment</th>
<th>Age, years</th>
<th>Baseline HBV DNA, log&lt;sub&gt;10&lt;/sub&gt; copies/ml</th>
<th>End follow-up HBV DNA, log&lt;sub&gt;10&lt;/sub&gt; copies/ml</th>
<th>Gender</th>
<th>CVR, PVR, rtR217, rtA219</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 (n=3)</td>
<td>+</td>
<td>2</td>
<td>31 and 40</td>
<td>6.1 and 8.7</td>
<td>3.6 and 7.8</td>
<td>1/1</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1</td>
<td>59</td>
<td>2.5</td>
<td>&lt;2.5</td>
<td>0/1</td>
<td>1/0</td>
</tr>
<tr>
<td>A2 (n=11)</td>
<td>+</td>
<td>7</td>
<td>47 (30–73)</td>
<td>7.3 (6.2–8.6)*</td>
<td>6.5 (5.4–8.1)*</td>
<td>5/2</td>
<td>7/0</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>4</td>
<td>48 (35–64)</td>
<td>7.4 (5.9–8.7)*</td>
<td>3.9 (3.3–4.3)*</td>
<td>2/2</td>
<td>4/0</td>
</tr>
<tr>
<td>D (n=25)</td>
<td>+</td>
<td>13</td>
<td>48 (32–74)*</td>
<td>6.9 (5.2–7.9)*</td>
<td>3.6 (&lt;2.5–8.1)*</td>
<td>10/3</td>
<td>8/5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>12</td>
<td>57 (37–59)*</td>
<td>7.3 (5.1–8.8)*</td>
<td>&lt;2.5 (&lt;2.5–6.6)*</td>
<td>6/6</td>
<td>9/3</td>
</tr>
<tr>
<td>F (n=2)</td>
<td>+</td>
<td>2</td>
<td>58 and 62</td>
<td>7.1 and 7.4</td>
<td>4.9 and 6.5</td>
<td>2/0</td>
<td>0/2</td>
</tr>
</tbody>
</table>

Baseline characteristics of the study population (41 patients) including demographics, serological and virological data and the number of patients (n) with complete virological response (CVR; end follow-up hepatitis B virus [HBV] DNA<2.5 log<sub>10</sub> copies/ml) or partial virological response (PVR; end follow-up HBV DNA<4 log<sub>10</sub> copies/ml) and variants, rtR217 and rtA219, grouped by HBV genotype and hepatitis B e antigen (HBeAg) status. *Mean (range). ADV, adefovir dipivoxil; CAH, chronic active hepatitis; LC, liver cirrhosis; 3TC, lamivudine; +, positive; -, negative.
Table 2. Predictive values of baseline parameters on complete and non-complete virological response

<table>
<thead>
<tr>
<th>Baseline parameter</th>
<th>Complete virological response</th>
<th>Non-complete virological response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predictive value, %</td>
<td>95% CI</td>
</tr>
<tr>
<td>HBeAg (+)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rtR1217</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HBV genotype A</td>
<td>93</td>
<td>66–99.9</td>
</tr>
</tbody>
</table>

Criteria of complete virological response (end follow-up hepatitis B virus [HBV] DNA <2.5 log_{10} copies/ml). Predictive values were calculated using \( \chi^2 \) and Fisher’s exact tests. Only significant (\( P<0.05 \)) and near significant results are listed. CI, confidence interval; HBeAg, hepatitis B e antigen; ND, no data; +, positive; -, negative.

Table 3. Predictive values of baseline parameters on partial and non-partial virological response

<table>
<thead>
<tr>
<th>Baseline parameter</th>
<th>Partial virological response</th>
<th>Non-partial virological response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predictive value, %</td>
<td>95% CI</td>
</tr>
<tr>
<td>HBeAg (-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rtR1217</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HBV genotype D</td>
<td>93</td>
<td>66–99.9</td>
</tr>
</tbody>
</table>

Criteria of partial virological response (end follow-up hepatitis B virus [HBV] DNA<4 copies/ml). Predictive values were calculated using \( \chi^2 \) and Fisher’s exact tests. Only significant (\( P<0.05 \)) and near significant results are listed. CI, confidence interval; HBeAg, hepatitis B e antigen; ND, no data; +, positive; -, negative.

Table 4. Statistical results of the time-to-event analysis showing the influence of baseline parameters on virological response

<table>
<thead>
<tr>
<th>Baseline parameter</th>
<th>Log-rank test/Kaplan–Meier</th>
<th>Multivariate Cox PH model</th>
<th>Univariate Cox PH model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \chi^2 )</td>
<td>P-value</td>
<td>HR</td>
</tr>
<tr>
<td>Complete virological response</td>
<td>HBeAg (+/-)</td>
<td>6.7</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>HBV genotype (D/A)</td>
<td>5.99</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>Rt217 (rtL217/rtR217)</td>
<td>6.86</td>
<td>0.01*</td>
</tr>
<tr>
<td></td>
<td>HBV DNA, log_{10} copies/ml</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Partial virological response</td>
<td>HBeAg (+/-)</td>
<td>17.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>HBV genotype (D/A)</td>
<td>3.35</td>
<td>0.06*</td>
</tr>
<tr>
<td></td>
<td>Rt217 (rtL217/rtR217)</td>
<td>4.04</td>
<td>0.04*</td>
</tr>
<tr>
<td></td>
<td>HBV DNA, log_{10} copies/ml</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Criteria of complete virological response (end of follow-up hepatitis B virus [HBV] DNA<2.5 log_{10} copies/ml) and partial virological response (end of follow-up HBV DNA<4 copies/ml). Only significant (\( P<0.05 \)) and near significant results are listed. The Fisher’s exact, \( \chi^2 \) and Mann–Whitney U tests yielded results similar to those obtained with the statistical tests presented in this table. *Variables showing a statistically significant association with virological response on the basis of complete or partial response criteria in the test. CI, confidence interval; HBeAg, hepatitis B e antigen; HR, hazard ratio; NC, not calculable; ND, no data; PH, proportional hazard; +, positive; -, negative.
Virological markers predictive of response to adefovir therapy

Figure 1. Kaplan–Meier curves for the cumulative probability of non-response, complete virological response and partial virological response

Kaplan–Meier curves for the cumulative probability of non-response, complete virological response (CVR; end follow-up hepatitis B virus (HBV) DNA < 2.5 log10 copies/ml) and partial virological response (PVR; end follow-up HBV DNA < 4 log10 copies/ml) and multivariate Cox model analysis for hepatitis B e antigen (HBeAg) status, HBV genotype A or D and rtL217R polymorphism. *HBeAg-negative/HBeAg-positive. †Genotype D/genotype A. ADV, adefovir dipivoxil; CI, confidence interval; HR, hazard ratio.
Analysis of HBV polymerase sequences before ADV treatment

No cases of HBV variants associated with ADV resistance (rtA181T/V, rtL233V and rtN236T) were observed at pretreatment. Comparison of the polymerase region sequences obtained with the consensus sequences deduced from the GenBank showed substitutions in 85 of the 200 amino acids analysed.

Statistical analysis of the polymerase sequence obtained was performed to investigate possible relationships between viral polymorphisms and response to ADV. Only the rtR217 polymorphism, which was detected in the 11 subgenotype A2 patients, was statistically associated with response: no patients with CVR (P=0.003) and three patients with PVR (P=0.002). For CVR, the presence of rtR217 showed a predictive value of 100% for non-response (end follow-up HBV DNA>2.5 log_{10} copies/ml). In the time-to-event analysis, the cumulative probability of non-response (Figure 1) was statistically different for rtR217L; however, no association was found with the Cox univariate model. Moreover, this polymorphism was not an independent predictor of response in the multivariate analysis (Table 2). For PVR, the presence of rtR217 showed a predictive value of 72.7% for non-response (end follow-up HBV DNA>4 log_{10} copies/ml). In the time-to-event analysis, the cumulative probability of non-response (Kaplan–Meier curves) was statistically different for the rtR217L polymorphism and the association was significant in the Cox univariate model. Nonetheless, this polymorphism was not an independent predictor of response in the multivariate analysis. Interestingly, the rtS219A variant, which was present in only one GenBank sequence, was observed at pretreatment in three subgenotype A2 patients who showed non-CVR (two of them had a PVR), although there was no statistical association.

Analysis of the HBV polymerase sequence during ADV treatment follow-up

Emergence of ADV drug-resistant variants at positions rt181 and/or rt236 was detected in 6 (14.6%) of the 41 patients. None of them had a CVR, but one with the rtA181V variant had a PVR. Comparison of sequences obtained during treatment and at pretreatment showed additional changes in 51 other positions. In nine of these positions, substitutions were only observed in non-CVR. Four substitutions (rtT128N, rtG152R, rtI162N and rtI163V) appeared in some patients and disappeared in others, and the remaining five substitutions (rtL115M, rtH121N, rtY151F, rtS176SN and rtS219A) only emerged during treatment. Variants at positions rtH121N, rtT128N, rtG152R and rtI162N were observed in four PVR patients (who showed non-CVR). Emergence of these variants was not associated with viral breakthrough. Remarkably, the rtS219 variant, detected at baseline in three non-CVR patients, emerged during therapy in three non-PVR patients (patient 1, subgenotype A1 and patients 6 and 8, subgenotype A2); the variant was detectable during treatment in all six patients.

Structural modelling of the HBV polymerase

In the first approach of selecting templates for homology modelling, none of the results obtained using the BLAST search appeared to be reliable for this purpose. Nonetheless, meticulous analyses of the alignments obtained showed some reliability on the basis of conservation of key residues related with catalysis and substrate recognition. Reviewing the hits, it was seen that all the proteins were reverse transcriptases from the Moloney murine leukaemia virus, HIV type-1 (HIV-1) and HIV type-2. These first hits were discarded because several residues in the crystal structure after the YMDD catalytic fingerprint had been missed. Finally 1LWO, a crystal structure of HIV-1 reverse transcriptase in complex with nevirapine, was used as the template [22]. It is known that some structural changes follow the binding of a ligand in the HIV-1 reverse transcriptase. Nonetheless, on the basis of the findings from previous studies, it is likely that structural similarity would be higher in the bound form of proteins than in the non-bound form when they share ligand specificity, particularly in the ligand-binding pocket [23]. The template structure shared 23% of identity and 40% of similarity with the HBV sequence. The region of similarity corresponded with residues 46–308 of the 1LWO structure and residues 166–308 of our HBV sequence. Various structural models were constructed and, after performing several checks based on energy and structural criteria, the best model according to the energy function from Modeller was used (Figure 2).

Superimposition of our model onto various protein structures analogous to HBV crystallized with various drug substrates showed a region in the binding pocket where certain residues were likely to be important in the binding model (Figure 2). Upon exploring the model and superimposition of homologous structures, it is interesting to note that rtY203 (in the YMDD motif catalytic loop) and rtV208 located in a β-sheet (βS1), were key residues in the binding pocket (particularly in HIV-1). Other residues that were also related in some way to ligand-binding and recognition were rtL199, rtV209, rtG210, rtY244, rtL246 and rtF248. Among all these positions, only rtL246 was non-conserved in relation to the HIV-1 reverse transcriptase structure, denoting the importance of these residues in recognition and binding.

On the basis of this homology model, residue 217, which could relate to ADV resistance [24], is located in the palm region of the HBV polymerase [25] at the edge of an α-helix (αH1) behind the substrate union site and
Virological markers predictive of response to adefovir therapy

Figure 2. Modelled hepatitis B virus reverse transcriptase superimposed onto the HIV reverse transcriptase. Modelled hepatitis B virus reverse transcriptase (green) superimposed onto the HIV reverse transcriptase (cyan) used as reference protein. In yellow, superposition of several ligands on several crystals deposited in the Protein Data Bank gives a general idea of the volume of the active site and the residues involved in binding, recognition and catalysis. The YMDD catalytic motif (residues 203–206) is shown in red sticks and the palm region in blue. Residues 215, 217 and 219, which are likely involved in resistance, are depicted in pink (underlined) and residues 233 and 236, which are directly related to adefovir dipivoxil resistance, in orange. Important superstructural motifs, including helices αH1 and αH2, βS1 and the coil region (CR) are shown in black.

is likely to be involved in some hydrophobic interaction with substrates. In addition, αH1 contains amino acids 214 and 215, where substitutions have been associated with resistance to ADV and 3TC [26], and amino acid 219 where, according to results of the present study, alterations (for example, rtS219A) might also be related with ADV resistance. Positions 233 and 236, reported to be involved in ADV resistance, are included in a coil region located behind the YMDD loop and are likely related to the pre-orientation of the catalytic loop.

Discussion

This study has identified several virological markers that could be useful as pretreatment predictors of response to ADV as monotherapy or in combination with 3TC. HBeAg-positive status was found to be an independent predictive factor of both CVR and PVR. Multivariate analyses showed that at 24 months of treatment, HBeAg-negative patients had a higher probability of virological response than HBeAg-positive patients (5-fold for CVR and 1.5-fold for PVR). These findings concur with results from our previous report, which included only patients with a PVR to ADV [12]; by contrast, results from an earlier study showed that ADV efficacy was similar regardless of HBeAg status [26]. This lack of agreement could be explained by the fact that the assessment of antiviral efficacy in the earlier study was based on a similar log10 HBV DNA decrease in both HBeAg-positive and -negative patients at 12 months. In addition, genotype A was found to be an independent factor indicating that patients will not achieve HBV DNA <2.5 log10 copies/ml (predictive value 93%). On multivariate analysis at 24 months of treatment, patients with genotype D had a sixfold higher probability of attaining HBV DNA <2.5 log10 copies/ml as compared with patients with genotype A. However, similar results were not found for HBV DNA <4 log10 copies/ml. These findings strongly suggest that HBeAg status and genotype are useful predictive factors of response to ADV as monotherapy or combined with 3TC. HBeAg-negative status together with genotype D had a predictive value of 92% for attaining HBV DNA <4 log10 copies/ml and 75% for HBV DNA <2.5 log10 copies/ml, whereas simultaneous detection of HBeAg-positive status and genotype A had a predictive value of 92% for not reaching HBV DNA <2.5 log10 copies/ml. Statistical analysis did not reveal any association between virological response and the use of ADV as monotherapy or in combination with 3TC, which agrees with a previous study [12]. However, in that previous study baseline HBV DNA status was independently associated with virological response (only tested for HBV DNA <4 log10 copies/ml), whereas in the present study, the associations with the two different criteria for response established only approached significance. This fact suggests the need for new studies with larger numbers of patients to test the association between baseline HBV DNA levels and virological response to ADV.

On analysis of the HBV polymerase sequence at pretreatment, only the rtL217R polymorphism was statistically associated with response to ADV. The presence of arginine at baseline (rtR217) showed a predictive value of 100% for not attaining HBV DNA <2.5 log10 copies/ml and 73% for not attaining HBV DNA <4 log10 copies/ml, indicating that these patients have some possibility of achieving only a partial response. These results agree with a previous observation in which rtR217 was detected in two of three patients with HIV–HBV coinfection and ADV resistance [27]. It is interesting to note that rtR217 is present in the A2 subgenotype, which is highly prevalent in Europe and the United States [18], as well as in our study population, where 11 of 14 genotype A patients were subgenotype A2. None of these 11 A2 patients achieved HBV DNA <2.5 log10 copies/ml and 3 of the 11 achieved values between 2.5 and 4 log10 copies/ml. These findings suggest that the high level of ADV resistance in our genotype A patients (all in subgenotype A2) is attributable to the presence of the rtR217 variant. Nonetheless, the limited number of subgenotype A1 patients in the present study did
not allow statistical confirmation of this hypothesis. The loss of association between HBV genotype and response to ADV, which has been reported previously, might derive from the fact that the association was based on a similar log_{10} HBV DNA decrease among the various HBV genotypes at 12 months of treatment or the fact that subgenotype analysis of genotype A was not performed [28]. It is worth noting that the rt217 position is included in a region of the viral polymerase (amino acid 215–226 that might mediate ADV resistance [24]. In fact, the rtV214A and rtQ215S variants have proven to be less sensitive to treatment with 3TC or ADV [26]. As for the other polymorphisms detected at pretreatment, most of them were found in both responders and non-responders; hence, they are likely to be unrelated to viral resistance. However, the rtS219 variant was only observed in three patients: one patient with complete non-response and two with values between 2.5 and 4 log_{10} copies/mL. Thus, it appears that only variants in positions 217 and 219 are implicated in the response to ADV.

In the analysis of emerging amino acid changes during ADV treatment, patients that did not attain HBV DNA<2.5 log_{10} copies/mL showed substitutions in only nine positions. Among these positions, positive selection of the rtS219A variant in four patients seems to be the only potentially relevant one. It is important to keep in mind that rtS219A, like rtL217R, is included in the 215–226 amino acid domain related with ADV resistance [24,26]. In fact, if the rtS219A variant were truly resistant to ADV (phenotypic confirmation is needed), its presence would explain the total non-response of patient 1 (subgenotype A1). The variants in positions 181, 217, 219 and 236 taken together would explain 15 of our 26 (58%) patients that did not attain HBV DNA<2.5 log_{10} copies/mL and 11 of our 18 (61%) patients that did not achieve HBV DNA<4 log_{10} copies/mL, whereas detection of the previously described and phenotypically confirmed variants 181, 233 and 236 would explain only 5 patients (20% CVR or 28% PVR) [1–3].

The three-dimensional structure of the HBV polymerase is not yet available. Therefore, a theoretical structural model of the HBV polymerase was developed in the present study to explain the possible mechanism of rtL217R resistance in ADV therapy and the role of subgenotype A2. Several homology models of the HBV polymerase structure using HIV-1 reverse transcriptase as template have been described and used for elucidating the molecular mechanism of resistance to different nucleos(t)ide analogues [25,28,29]. According to our structural model, residue 217 is located in the palm region of the HBV polymerase (nomenclature of Das et al. [25] at the edge of an α-helix structure (αH1 in Figure 2) behind the substrate union site. The αH1 structure also contains amino acid 219, whose alterations (for example, rtS219A) might also be related with ADV resistance according to the results of the present study. It is worth noting that positions 217 and 219 are part of domain 215–226, previously described as being related to ADV resistance [24,26]. Positions 233 and 236 in our model, which were associated with ADV resistance, are included in a coil region located behind the YMDD catalytic loop. Structural elements, αH1 and βS1 are joined with a second α-helix (αH2), containing positions 180 and 181, and the coil region, which includes positions 233 and 236, seems to build a pocket for nucleotide substrates of the HBV polymerase. rtL217 allows hydrophobic interaction, whereas substitution of leucine to arginine (a positively charged amino acid) might disrupt this interaction, altering the packaging of the binding site pocket and producing a change in the affinity for ADV. This hypothesis would explain the low activity of ADV against HBV subgenotype A2, which carries rtR217; none of the patients with this profile attained a CVR. Nonetheless, this variant should not be classified as ADV-resistant because three subgenotype A2 patients were PVR; hence it would be more appropriate to consider it as a ‘low-sensitivity variant’ to ADV therapy, perhaps requiring higher drug doses. With regard to this point, increasing the ADV dose might improve virological response. In a recent study in five ADV non-responders, Hezode et al. [8] showed that a dose increase from 10 to 20 mg daily enhances ADV efficacy. Further studies including subgenotyping analysis in a large number of patients are needed to test our hypothesis.

The results of the present study might be useful when establishing ADV treatment. From the practical viewpoint, pretreatment analysis of position 217 could be the first parameter to consider. Thus, when rtR217 is detected, non-response to ADV can be predicted with a high probability at both response criteria. Moreover, when rtL217 is detected and the more restrictive response criteria (CVR) is applied, HBeAg-negative patients would have a 77% probability of response to ADV treatment and HBeAg-positive patients a 70.6% probability of non-response. In geographical areas where subgenotype A2 represents the greatest proportion of genotype A cases, analysis of position 217 can be substituted for genotype determination with similar results (93% predictive value of non-response for genotype A). When HBV genotype was used in our patients as the first determination followed by HBeAg status determination in non-A genotypes (mainly genotype D), HBeAg-negative patients showed a 75% probability for response to ADV therapy. In conclusion, this study describes the virological characteristics, HBeAg status and HBV genotype in pretreatment samples that are independently associated with antiviral response to ADV therapy. These factors,
together with the rtL217R polymorphism, are potentially useful as predictive factors of response or nonresponse to ADV. Development of a structural model of the HBV polymerase allowed formulation of a hypothesis for the molecular mechanism causing resistance of the rtR217 variant to ADV. However, phenotypic experiments must be applied to demonstrate the true affinity of the rtR217 and rtA219 variants for ADV and more complex computational models should be developed to test the effect of rtL217R substitution in the HBV polymerase substrate pocket structure.

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Disclosure statement

The authors declare no competing interests.

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