Frequency and Mutation Patterns of Resistance in Patients with Chronic Hepatitis B Infection Treated with Nucleos(t)ide Analogs in Add-On and Switch Strategies

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ABSTRACT

Background: Treatment for chronic hepatitis B (CHB) has improved over the last 10 years mainly due to the development of effective oral antiviral agents [nucleoside/nucleotide analogs (NUCs)].

Objectives: The aim of the present study is to identify the frequency and major patterns of resistance to the hepatitis B virus (HBV) in a Turkish population of CHB patients treated with NUCs using add-on and switch therapy strategies.

Patients and Methods: The investigation involved a total of 194 patients (88 were treated using add-on therapy, and 106 were treated using switch therapy). We analyzed the HBV polymerase gene by amplification and direct sequencing procedures.

Results: Primary drug-resistance mutations were detected in 84 patients (43%; 42 in add-on therapy, and 42 in switch therapy) taking lamivudine (LAM), 10 patients (5%; 6 in add-on therapy, and 4 in switch therapy) taking entecavir (ETV), and 16 patients (8%; 8 in add-on therapy, and 8 in switch therapy) taking adefovir (ADV). The most common LAM and ETV resistance mutations were rtM204I/V, rtL180M and rtT184A/I/S, respectively, while rtA181T/V and rtN236T substitutions were the most frequently observed ADV resistance mutations.

Conclusions: Patients with CHB who developed NUC resistance were managed using 2 different rescue strategies. The frequency and mutation pattern of resistance were similar in patients treated with add-on and switch strategies. These findings may be helpful in the management of rescue strategies in LAM-resistant patients.

Implication for health policy/practice/research/medical education:
Authors revealed of major drug resistance patterns and their frequencies in add-on and switch strategies in treatment of chronic hepatitis B virus. This article is an helpful for clinicians involved in the management of rescue strategies in lamivudine-resistant patients in chronic hepatitis B.

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Over the last 10 years mainly due to the development of effective oral antiviral agents [nucleoside/nucleotide analogs (NUCs)] (3). NUCs used in hepatitis B virus (HBV) therapy belong to the following 3 classes:

- L-nucleosides: lamivudine (LAM), telbivudine (LdT), and emtricitabine; Deoxyguanosine analogs: entecavir (ETV); Acyclic nucleoside phosphonates: adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF).

Currently, LAM, ADV, ETV, TDF, and LdT have been li-
3. Patients and Methods

3.1. Patients

This study was designed as a retrospective molecular study based on different rescue strategies implemented in the treatment of CHB patients with NUCs. All patients were consecutively enrolled. Molecular analysis was performed from March 2007 to November 2010 at the Kocaeli University Hospital. Patients with CHB were assigned to 2 groups: the first group (n = 88) was treated with NUCs using an add-on strategy (adding another drug effective against the drug-resistant mutant in patients from the Gastroenterology Department) after developing LAM resistance; the second group (n = 106) was treated with NUCs using a switch strategy (switching to a new antiviral agent after the development of resistance in patients from the Infectious Diseases Department). LAM (Zeffix, 100 mg/day; Glaxo Wellcome Laboratories, Middlesex, UK), ADV (Hepsera, 10 mg/day; Gilead Sciences Inc., Foster City, USA), ETV (Baraclude, 0.5 mg/day or 1 mg/day; Bristol-Myers Squibb Company, Princeton, USA), and TDF (Viread, 245 mg/day; Gilead Sciences Inc., Foster City, USA) were the oral anti-HBV drugs used in this study. Clinical and laboratory characteristics of patients in each group are shown in Table 1. Laboratory results revealed that all patients were infected with HBV, the virus is characterized by intermediate levels (2%-7%) of endemicity (17). Moreover, studies conducted in Turkish patients having treated or untreated CHB infections indicated that HBV drug resistance is frequently mediated by rtM204V (YVDD variant) and rtM204I (YIDD variant) mutations, and rtM204S (YSDD variant) mutations with or without compensatory mutations such as rtV173L and rtI180M are also found, but occur much less frequently (18-21).

2. Objectives

In the present study, 2 different treatment strategies were applied for both hepatitis B e antigen (HBeAg)-positive and HBeAg-negative CHB patients. The aim of the study is to determine the patterns and frequency of primary and compensatory mutations in patients undergoing long-term NUC treatment using add-on and switch therapy strategies.
3.2. DNA Isolation and Real-Time PCR

HBV DNA was isolated from serum samples with a biorobot workstation using magnetic-particle technology (NucliSENS-easyMag, bioMérieux, Boxtel, Holland). HBV DNA was detected and quantified using a commercial real-time PCR assay and platform (Jontek Biotechnology Inc., Istanbul, Turkey; and the iCycler iQ5 detection system, Bio-Rad Laboratories Inc., California, USA).

3.3. Sequencing of the HBV Polymerase Gene Region

Briefly, a pair of primers was designed (forward: 5’-TCGTTGGACTTCTCCAATTT-3’ and reverse: 5’-CGTGA-CAGACTTTCCAATCTAA-3’) for amplification of the HBV polymerase region. PCR conditions were 95°C for 15 min, followed by 45 cycles consisting of 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s. The final primer concentration was 0.3 μM, and the HBV amplicon size was 742 bp. All PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and directly sequenced on ABI PRISM 310 Genetic Analyzer equipment using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, USA). For cycle sequencing, we used the following thermal protocol: 35 cycles consisting of 95°C for 20 s, 50°C for 25 s, and finally 60°C for 2 min. The reverse primer was used as the sequencing primer at a final concentration of 0.5 μM. Electropherogram-obtained sequences were assembled using Vector NTI v5.1 (InforMax, Invitrogen Life Science Software, Frederick, MD, USA).

3.4. Determination of HBV Genotype

HBV genotypes were determined using genotyping tools from the National Center for Biotechnology Information (NCBI, U.S. National Library of Medicine, Bethesda, USA, http://www.ncbi.nih.gov/projects/genotyping/formpage.cgl) to identify the genotype based on the viral nucleotide sequences. This genotyping tool works by using a BLAST search to compare a query sequence to a set of reference sequences for known genotypes (23). The Genofor/Arevir geno2pheno drug resistance tool (Center of Advanced European Studies and Research, Bonn-Germany, http://www.geno2pheno.org/) was used for HBV subgenotyping.

3.5. Determination of Polymerase and Surface Gene Mutations

Data accumulated by direct sequencing were analyzed either manually or using the geno2pheno tool. The Genofor/Arevir geno2pheno drug resistance tool for HBV is a database that is specifically designed for rapid computer-assisted virtual phenotyping of HBV, and accepts genome (nucleic acid) sequences as input. This geno2pheno program searches for homology between input sequences and other sequences already stored in its database, which includes relevant clinical data for drug resistances and surface gene mutations (3). The tool searches for HBV drug resistance mutations in the rt domain of the polymerase at amino acid positions 80, 169, 173, 180, 181, 184, 194, 202, 204, 215, 233, 236, and 250. However, we also manually analyzed rt amino acid substitutions at positions 84, 85, 214, 237, and 238 (6). The overlapping S-gene segment was evaluated using the geno2pheno tool for 5 amino acid substitutions at positions 137, 141, 144, 145, and 147 and using manual search at positions 121, 135, 139, 140, 142, 146, 148, 149, 151, 152, 153, 155, 156, and 157 (24).

3.6. Statistical Analysis

Age, HBV DNA load, alanine aminotransferase (ALT) levels, and aspartate aminotransferase (AST) levels were considered as the numerical values, while gender and HBeAg positivity represented the categorical variables. The significance of differences between 2 numeric variables was compared using Mann-Whitney U test. The significance of differences between 2 proportions was measured using Pearson Chi-square test. P values that were ≤0.05 were considered statistically significant. Statistical analyses were conducted using SPSS software version 13.0.0 (SPSS Inc., IL, USA) for Windows.

Demographic data and clinical features for each of the study groups are summarized in Table 1. Mutations in the HBV polymerase gene known to confer resistance to NUCs were found in 122 out of 194 patients (63%). Of these 122 patients, 68 (56%) were in the add-on therapy group and 54 (45%) were in the switch therapy group. Four different LAM resistance patterns were identified in 84 out of 194 patients (43%): baseline primary mutation (rtM204I/V, n = 26), primary mutation with the compensatory mutations rtL80I/V (n = 16) or rtL80M (n = 24), triple mutant (rtM204I/V + rtV173L + rtL80M or rtA194G, n = 12), and the single mutation rtL80M (n = 6). The most frequent mutations detected in response to LAM treatment were rtM204I/V and rtL80M (Table 2). LAM-resistance mutations (including ETV resistance mutations) were identified in 48 out of 88 patients (55%) in the add-on therapy group (median LAM therapy duration, 28.7 months, with an LAM + ADV combination therapy duration of 23.3 months) and in 46 out of 106 patients (43%) in the switch therapy group (median therapy duration, 24.8 months). This difference was not statistically significant (P > 0.05) (Tables 1 and 2). LdT-resistance patterns were identified in 60 out of 194 patients (31%), and there were no significant differences in the frequencies of these mutations between the add-on and switch therapy groups (P > 0.05). The observed patterns of LdT resistance included baseline primary mutations (rtM204I, n = 24) associated with the compensatory mutations rtL80I/V or rtL80M (n = 28) and triple mutants (rtM204I + rtV173L + rtL80M or rtL80I/V, n = 8; Table 1). The ADV-associated mutations rtA181T/V, rtN236T, and rtA181T + rtN236T were detected in 6, 8, and 2 patients, respectively (for a total of 16 out of 194 patients, or 8%). Primary ADV-resistance mutations (i.e. N236T) were most
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Advocated by Yan M et al., frequently detected. ADV-resistance mutations were identified in 8 out of 88 patients (9%) in the add-on therapy group (median ADV therapy duration, LAM + ADV: 23.3, ADV + ETV: 19.5 months) and in 8 out of 106 patients (7.5%) in the switch group (median therapy duration, 13.1 months). This difference was not statistically significant (P > 0.05; Tables 1 and 2). Single compensatory mutations were not detected in the switch therapy group; however, rtQ215H/P/S ± rtV214A/P mutations were found in 12 out of 88 patients (14%) in the add-on therapy group, and this difference was significant (P < 0.01).

The ETV-resistance mutation rtM204I/V + rtL180M + rtT184A/I/S or rtS202C was found in 10 out of 194 patients (5%). Of these 10 patients, 6 out of 88 (7%) were in the add-on therapy group (median ETV therapy duration as ADV + ETV combination, 19.5 months) and 4 out of 106 (4%) were in the switch therapy group (median ETV therapy duration, 11.4 months). There was no significant difference in ETV drug resistance mutations between the 2 groups (P > 0.05; Tables 1 and 2).

Mutations conferring resistance to TDF were not detected. In 1 patient in the add-on therapy group, a multi-drug-resistant HBV strain was detected during combination therapy with LAM and ADV. Eight patients also had changes in the amino acid sequence of the overlapped S-gene segment. Two patients in the add-on therapy group had sC137G amino acid substitutions (with rtL80M + rtM204I mutations), 2 patients in the switch therapy group had sG145R mutations (with rtN236T mutations), and 4 patients in the switch therapy group had sD144E mutations (1 patient with rtL80M + rtM204I mutations and 3 patients with rtQ215S mutations).

Direct sequencing results revealed that all patients had HBV genotype D. Subgenotype D1 was identified as the HBV genotype in 74 out of 88 patients (84%) and 82 out of 106 patients (77%) in the add-on and switch therapy groups.

### Table 1. Demographic Data and Clinical Features of Patients in Each Study Group

| Add-on Strategy, (n = 88) | Switch Strategy, (n = 106) |
|---------------------------|---------------------------|
| **Male, No. (%)**         |                           |
|                           | 66 (75)                   |
|                           | 70 (66)                   |
| **Age, median y (range)** |                           |
|                           | 45 (13-68)                |
|                           | 38 (16-61)                |
| **HBeAg positive, No. (%)** |                      |
|                           | 30 (34)                   |
|                           | 36 (33.9)                 |
| **ALT**<sup>b</sup>, median U/L (range) |           |
|                           | 68 (16-537)               |
|                           | 84 (12-1082)              |
| **AST**<sup>b</sup>, median U/L (range) |               |
|                           | 61 (14-709)               |
|                           | 55 (13-579)               |
| **HBV DNA, median log<sub>10</sub> IU/mL (range)** |         |
|                           | 4.4 (2.0-6.0)             |
|                           | 3.8 (2.0-6.1)             |
| **HBV subgenotype D, No. (%)** |               |
| D1                        | 74 (84)                   |
|                           | 82 (77)                   |
| D2                        | 12 (14)                   |
|                           | 12 (11)                   |
| D3                        | 2 (2)                     |
|                           | 10 (10)                   |
| D4                        | 0 (0)                     |
|                           | 2 (2)                     |
| **History of chronic hepatitis B infection** |                       |
| Patients in the immune-tolerant phase | 24                      |
|                           | 22                       |
| Patients in the immune-reactive phase | 6                      |
|                           | 14                       |
| Patients with Knodell fibrosis scores | 58                      |
|                           | 70                       |
| Patients without biopsy | 48                       |
|                           | 34                       |
| **Therapy status**<sup>c</sup> |                   |
| LAM<sup>b</sup> → LAM + ADV<sup>b</sup> | 74                      |
|                           | -                        |
| ADV → ADV + ETV<sup>b</sup> | 14                      |
|                           | -                        |
| LAM → ADV + ETV            | -                        |
|                           | 12                       |
| LAM → ADV + TDF<sup>b</sup> | -                        |
|                           | 10                       |
| LAM → ETV                  | -                        |
|                           | 24                       |
| ADV → ETV                  | -                        |
|                           | 30                       |
| **Treatment duration, median mon (range)** |                  |
| LAM                        | 28.7 (3-60)              |
| LAM + ADV                  | 23.3 (6-48)              |
| ADV                        | -                        |
| ADV + ETV                  | 16 (6-22)                |
| ETV                        | -                        |
|                           | 11.4 (6-24)              |

<sup>a</sup> Serological markers of all patients were found to be negative for hepatitis C virus and hepatitis D virus.

<sup>b</sup> Abbreviations: ADV, adefovir dipivoxil; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ETV, entecavir; LAM, lamivudine; TDF, tenofovir disoproxil fumarate.

<sup>c</sup> The combination of NUCs used was selected according to the emergence of drug resistance (primary or compensatory resistance) or clinical and/or virological breakthrough.
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5. Discussion

The long-term use of monotherapy drugs in CHB, and LAM in particular, is frequently associated with the development of drug resistance. Ideally, antiviral drugs used in combination should be carefully chosen to have different mechanism or sites of action and should act in an additive or synergistic fashion (5, 25). According to a recently published study, time-limited add-on strategies do not provide benefits over switch strategies with respect to the emergence of ADV-resistant mutants in LAM-resistant CHB patients (26). In contrast, add-on ADV therapy was found to be more effective and longer lasting than ETV as a rescue therapy in patients with LAM-resistant mutations who required long-term antiviral treatment (27). According to EASL clinical and practice guidelines for cases of drug resistance, an appropriate rescue therapy should be initiated with the most effective NUCs and with minimal risk of inducing multiple drug-resistant strains. Therefore, the only effective strategy is the addition of a second drug that does not display cross-resistance (4). The results of this study demonstrated that add-on and switch therapy strate-
gies using NUCs to treat CHB conferred similar major drug-resistance patterns at approximately the same frequency, with the exception of compensatory mutations such as rtQ215S. Major drug-resistance patterns resulting from LAM and LdT treatment occurred more frequently than ADV- or ETV-resistance patterns. Resistance to LAM and LdT may develop when using these agents in long-term treatment (median treatment duration with LAM: 28.7 months in the add-on group and 24.8 months in the switch group; Table 1). In fact, studies have shown that the frequency of viral resistance progressively increases from 10%–27% at the initial diagnosis to 37%–48% by the end of the second year of LAM monotherapy (3). In our study, the rtM204V mutation was detected as a single mutation in only 1 patient under monotherapy. This mutation is usually not detected as a single mutation, but instead is usually found in combination with rtL180M (5). Replication defects resulting from NUC treatment can be partly compensated for by selection for compensatory mutations (7, 28). The rtL180M mutation was the most common compensatory mutation in our study. In vitro studies have demonstrated that this mutation alone is insufficient to result in LAM resistance; however, this mutation augments both viral replication and LAM resistance in the context of rtM204I/V mutations. The rtV173L HBV mutation occurs in 9% of LAM-resistant patients (5) and serves to further increase the replication capacity of poorly replicating LAM-resistant HBV (2). Mutation at codon rtL180I/V may also be an alternative compensatory mutation to rtL180M in HBV genomes encoding the rtM204I mutation, and this mutation may act in a manner similar to rtQ215S. In the current study, mutations at rtQ215S were only observed in the add-on therapy group in 12 patients (14%), while previous studies have described the rtQ215S mutation as a polymorphism that is detected in ~12% of patients treated with LAM (5). Substitutions at rtQ215 also occur during ADV therapy (6). However, Olyaei et al. demonstrated no association between rtQ215 mutations and clinical complications in patients infected with HBV genotype D (29). Accordingly, in our previously published report, we found that rtQ215A/H/P/S substitutions could be detected as naturally occurring mutations in treatment-naive patients with CHB (30). Furthermore, rtQ215H/P/S substitutions occur as dominant compensatory mutations in treatment-naive hemodialysis patients infected with HBV genotype D (unpublished data). However, in vitro studies suggest that rtQ215 substitutions impair neither viral replication efficiency nor susceptibility to LAM or ADV (29). LdT, another nucleoside analog, also selects for mutations in the YMDD motif, similar to mutations conferring resistance to LAM, and is not expected to be effective in LAM-resistant patients. Resistance to LdT also begins to emerge in the first year of treatment and progressively increases during the second year of therapy (3). LdT was not used in either therapy strategy implemented in this study. Nevertheless, we included the frequency and patterns of LdT drug resistance in Table 2, according to EASL clinical and practice guidelines (4).

The rtA181T HBV mutation is a major mutation pattern associated with LAM resistance, but selected for during ADV treatment and during the development of resistance to combination (ADV + LAM) therapy in the absence of mutations at rtM204I/V (5). In this study, rtA181T mutations were detected in 6 patients who did not also carry the rtM204I/V mutation. ADV drug resistance detected in this study was found to be associated with mutations at rtN236T and rtA181T. Further, rtQ215S and rtV214A mutations were compensatory mutations arising from ADV treatment (10). However, while the rtN236T substitution does not significantly affect sensitivity to LAM, rtA181T/V and rtQ215S/rtV214A mutations confer partial cross-resistance to LAM (31).

Resistance to ETV is associated with 2 different mutation profiles, both of which include LAM-resistance mutations (5, 9, 32). High levels of ETV resistance resulted from dual rtM250V and rtI69T mutations, and we detected another profile, the rtM204I/V + rtL180M + rtT184A/I/S or rtS202C triple mutation, as an ETV-resistant mutation in both NUC therapy strategies. On the other hand, 24 patients receiving LAM treatment in the switch therapy group were switched to ETV therapy at 1 mg daily (Table 1), and while these patients had no LAM-resistance mutations, they were refractory to LAM therapy. In this group of LAM-refractory patients, the emergence of ETV resistance occurred more frequently than was the case with NUC treatment-naive patients (33). ETV and TDF are both potent HBV inhibitors and have a high barrier to resistance. Thus, they can be confidently used as first-line monotherapies (4).

HBV strains that are resistant to at least 2 anti-HBV agents from different NUC subclasses without cross-resistance profiles are defined as multidrug-resistant strains (3). These multidrug-resistant strains are more likely to develop additional mutations with sequential therapy (34-36). In the current study, we found 1 patient with dual ADV (rtN236T + rtQ215H) and LAM (rtM204V + rtL180M + rtV173L) resistance, detected in different serum samples from the same patient. Recently, the emergence of the first multidrug-resistant HBV strain arising from sequential oral anti-HBV therapies was documented (35). Nevertheless, there is limited in vivo data demonstrating resistance to multiple NUCs (3).

Because of overlapping open reading frames of the HBV polymerase and the HBsAg, drug resistant mutations in the HBV polymerase can have a direct impact on the nature of the HBsAg and its function (28, 37). Mutations in and around the major neutralization domain of HBV, known as the ‘a’ determinant, may result in decreased affinity of the HBsAg to anti-HBs and cause diagnostic problems and/or failure to prevent infections by vaccination or HB immunoglobulin (7, 38). Studies have shown that LAM-resistant HBV (harboring the rtV173L + rtL180M + rtM204V triple mutation) has significantly reduced an-

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Several studies have shown that HBV genotype D represents almost all isolates from the Turkish HBV patient population (33, 39, 42, 43). However, we recently documented a case of HBV genotype A in Turkish patient (44). The present study demonstrated that HBV genotype D is still dominant among Turkish CHB-infected patients. Because of the dominance of genotype D, it is difficult to evaluate NUC resistance in relation to the different genotypes of HBV. A limited number of reports have demonstrated the relationship between HBV genotype and the response to antiviral therapy with LAM (15). Zollner et al. reported that the mutational pattern during the selection of LAM-resistant HBV strains differs between genotypes A and D. However, EASL clinical practice guidelines have not yet supported any relationship between the HBV genotype and response to NUC therapy (4). To date, there is little data describing the subgenotyping of HBV in Turkish patients. In the present study, HBV pol gene sequences isolated from Turkish patients revealed that subgenotype D1 constitutes the majority of genotype D circulating in Turkey. We also demonstrated the presence of subgenotypes D2, D3, and D4; however, based on pol gene sequencing, HBV subgenotype D1 was predominant (84%) and subgenotypes D2, D3, and D4 were found in only 10%, 5%, and 0.2% of Turkish patients with CHB (n = 442), respectively (45). In contrast, other groups have shown that subgenotype D2 is the predominant HBV subgenotype in Turkish patients (46-48). While both these studies use pre-S gene amplification along with restriction fragment length polymorphism techniques, the discrepancies in the reported results are probably due to differences in methodology. 

In conclusion, CHB is a disease that can develop progressive resistance to NUCs by mutations in the HBV polymerase gene. Therefore, it is necessary to use effective therapeutic strategies to manage drug resistance. However, we did not detect a significant difference in the emergence of major drug resistance patterns and their frequencies when add-on and switch strategies were implemented. These findings may prove to be useful in the management of rescue strategies in LAM-resistant patients.

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