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PREVALENCE AND MUTATIONAL PATTERNS OF LAMIVUDINE-RESISTANT HBV STRAINS IN CHRONICALLY INFECTED SERBIAN PATIENTS

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Abstract - This study aimed to determine the prevalence and mutational patterns of lamivudine-resistant hepatitis B virus (HBV) strains in chronically infected Serbian patients. The study included 154 patients on long-term lamivudine monotherapy. Resistance-associated mutations were identified by direct sequencing of the S/P gene. The genotypic resistance to lamivudine was confirmed in 54.5% of patients. Three primary resistance-associated mutations were found: rtM204V (55.9%), rtM204I (40.5%), rtA181T (3.6%) and two compensatory mutations rtV173L (17.8%) and rtL180M (67.8%). Seven mutational patterns were discovered with rtL180M+rtM204V being dominant (41.6%). The presence of resistance-associated mutations was correlated to the older age of patients, the presence of clinically relevant HBsAg mutations and higher values of viral load. No correlation with HBV genotypes, subgenotypes or HBsAg subtypes was observed. High prevalence of resistance supports the use of genotypic testing in monitoring patients on lamivudine therapy and selecting those who would benefit from therapy with newly developed nucleos(t)ide analogs.

Key words: Hepatitis B virus (HBV), lamivudine, resistance, mutations

INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of acute and chronic hepatitis and of its long-term complications. About 350 million people are currently infected with HBV worldwide and approximately 1 million die of liver failure, cirrhosis and hepatocellular carcinoma (HCC) each year (Lavanchy, 2005, Weber et al., 2011).

It has been shown that the risk of liver disease progression in patients with chronic hepatitis is associated with elevated HBV DNA levels (Chen et al., 2006, Iloeje et al., 2006). Therefore, the goal of therapy for chronic hepatitis patients is to delay or prevent the progression of liver disease by suppressing long-term HBV DNA replication (Negro, 2011). The development and introduction of nucleos(t)ide analogs (NA) has played a major part in the progress of chronic hepatitis B treatment that has occurred over the past decade. Since the introduction of lamivudine (LMV) in 1998, new drugs have been developed and today a large number of patients is on long-term NA therapy. Currently, lamivudine, adefovir, entecavir, telbivudine and tenofovir have been approved worldwide for the treatment of chronic B hepatitis (Chien and Liaw, 2008).

In Serbia, tenofovir, the representative of the latest generation of NA, is currently available for the treatment of HBV infection, but until recently, all patients requiring treatment were on long-term lamivudine

monotherapy. Lamivudine is a deoxycytidine analog that inhibits HBV DNA synthesis through chain termination. It has been shown to be effective in reducing HBV DNA levels, normalizing serum transaminases and improving histological indices in patients with both HBeAg positive and negative disease (Lok and McMahon, 2004). The major drawback of lamivudine treatment is the high rate of viral resistance related to mutations. Mutations conferring resistance to NA are located in the reverse transcriptase (RT) domain of the HBV polymerase gene. The kinetics of replacement of the wild-type virus in liver cells by a dominant mutant is slow because resistant mutants mainly infect new uninfected cells. LMV resistance increases progressively over the course of treatment; 14-36% after 1 year of treatment, increasing to 38%, 49% and 66% after 2, 3, and 4 years of treatment, respectively (Lau et al., 2000, Liaw et al., 2000, Lai et al. 2003).

Most lamivudine resistant HBV strains are characterized by an exchange of the methionine by an isoleucine or a valine (rtM204I/V mutants) in the YMDD motif, the active site within RT (aa203-206). LMV resistance by the HBV mutations at position 204 is due to changes in the van der Waals forces (or the intramolecular interactions) that results in the repositioning of the antiviral agent as well as steric hindrance between the sulfur atom in LMV and the rtM204I/V side chain (Bartholomeusz and Locarnini 2006).

The change at position 204 is usually not detected as a single mutation but in combination with different secondary or compensatory mutations. The role of compensatory mutations is to restore functional defects in viral polymerase activity (i.e., replication fitness) associated with primary drug resistance (Lok et al., 2007a). Five major compensatory mutations have so far been reported (rtL80I, rtI169T, rtV173L, rtL180M, rtT184S) and they are associated with primary mutations in nine major patterns (Bartholomeusz and Locarnini, 2006). As an exception, one of the patterns includes the existence of a single primary mutation (rtA181T) outside of the YMDD motif that was first detected in a patient with increasing HBV DNA levels during LMV treatment without the classical LMV resistance (Yeh et al., 2000).

The aims of the present study were: (1) to determine the prevalence and mutational patterns of lamivudine-resistant strains in Serbian patients on long-term therapy, and (2) to correlate the presence of genotypic resistance to lamivudine with demographic and other virological parameters.

MATERIALS AND METHODS

Patients

The study comprised 154 plasma samples, collected over a 6-year period (2006-2012) from patients with chronic hepatitis B treated at the Clinics of Infectious and Tropical Diseases in Belgrade. The diagnosis was based on HBsAg positivity for >6 months and histopathological verification by liver biopsy. One hundred and eighteen patients were male (76.6 %) and 36 were female (23.4%). Age ranged from 9 to 76 years (mean: 44.4±15.5 years). All patients were on lamivudine monotherapy for at least two years. Informed consent for participating in this study was obtained from all patients and the study was approved by the Ethics Committee of Faculty of Medicine, University of Belgrade No. 29/VI-12.

Isolation and amplification of viral DNA

Isolation of viral DNA was carried out using a QIAmp Blood Mini Kit (QIAGEN GmBH, Hilden, Germany) according to the manufacturer's instructions. HBV DNA was amplified using nested PCR that targeted a 487-bp region of the S/P gene. Amplification was performed using the following primers: 252 (5'-AGACTCGTGGTGGACTTCTCT-3')/1309 (5'-AGAATGTTTGCTCCAGACC-3') as external primers and 377 (5'-GGATGTGTCTGCGGCGTTT-3')/840 (5'-ACCCCATCTTTTTGTTTTGTTAGG-3') as internal primers, as described previously (Zollner et al., 2002). After both reactions, a 487-bp fragment was obtained and detected by ethidium bromide staining in an agarose gel.

DNA purification and sequencing

Nested-PCR products were purified using the QIAGEN MinElute Purification Kit (QIAGEN GmBH, Hilden, Germany), according to manufacturer's instructions. For cycle sequencing reactions, internal PCR primers and a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used. Sequencing was carried out in an automatic sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Sense and antisense strands were sequenced and compared. A 444-nt fragment of the S gene (nt 394-837), which comprised codons 80-227 of the HBsAg molecule, was selected for analysis.

Identification of lamivudine-resistance mutations and HBsAg mutations

For identification of lamivudine-resistance mutations and HBsAg mutations, all obtained sequences were compared with a reference wild-type sequence of the same genotype in BioEdit software (Hall, 1999). Amino acid substitutions were discovered by applying open reading frames for either P or S gene.

Identification of HBV genotypes, subgenotypes, HBsAg subtypes

The viral genotype and subgenotype was identified by phylogenetic analysis. The nucleotide sequences were aligned pairwise using ClustalW, and a phylogenetic tree was constructed by the neighbor-joining method and Kimura two-parameter algorithm in MEGA software (version 5.05) (Tamura et al., 2011). Statistical significance of phylogeny was estimated by bootstrap analysis with 1 000 pseudoreplicate datasets.

The HBsAg subtype was deduced from the sequence of the same S gene region used for genotyping, based on identifying amino acids at positions 122 (Lys/Arg for d/y determinants), 160 (Lys/Arg for w/r determinants), 127 (Pro/Thr/Leu-Ile for w2/w3/w4), and in the case of Arg122 Pro127 Lys160, also at positions 159 (Ala/not Ala for ayw1/ayw2 and ayw4) and 140 (not Ser/Ser for ayw2/ayw4).

HBV DNA Quantitation

For determination of HBV DNA level in plasma, an in-house real-time PCR protocol was performed, using pre-designed primers and probes from Applied Biosystems, TaqMan Universal PCR Master Mix (Applied Biosystems) and 4 whole genome standards 10^3-10^6 copies/μL (Clonit S.r.I., Milano, Italy). All HBV DNA values are reported in IU/ml; values given as copies/ml were converted to IU/ml by division by 5.6. For the presentation of HBV DNA levels logarithmic scale was used.

Statistical analysis

Data statistical analysis was performed using the SPSS software package 20.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Chi-squared test, Student t-test, Mann-Whitney U-test and Spearman's rank correlation test were used for comparisons and correlations of groups.

RESULTS

Plasma samples of 154 patients on long-term lamivudine therapy were tested for the presence of HBV DNA. The majority of patients were positive for HBV DNA – 132 (85.7%), while 22 (14.3%) did not have detectable HBV in peripheral blood. Positive samples were tested by real-time PCR for determination of viral load. Viral load values varied from 3.65 to 10 log IU/mL with the mean value of 6.04±1.86 log IU/mL.

For determination of viral genotypes and subgenotypes, a phylogenetic tree (not shown) was constructed based on sequences of the 444-nt fragment of the S/P gene (nt 394-837). Two HBV genotypes were encountered: D in 114 (86.4%) and A in 18 (13.6%) patients. All genotype A isolates clustered with the representative sequence for European A2 subgenotype in the phylogenetic tree, whereas genotype D isolates belonged to three different subgenotypes: 8

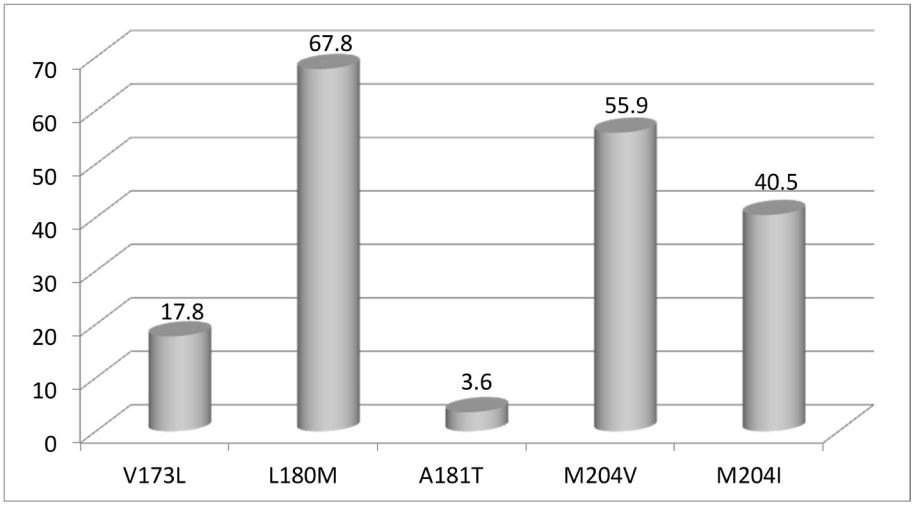

Fig.1. Frequencies (%) of discovered resistance-associated mutations.

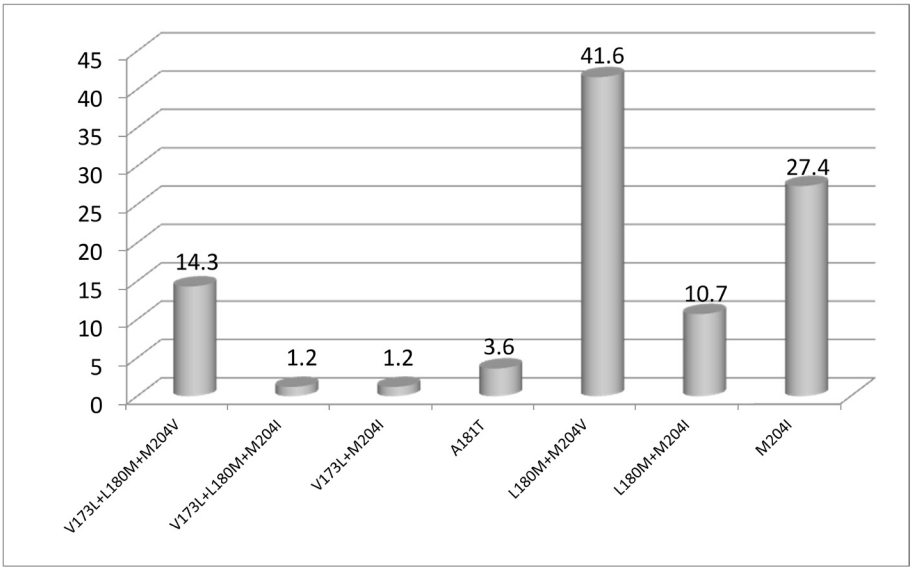

Fig.2. Frequencies (%) of mutational patterns in lamivudine-resistant isolates.

to D1 (6.1%), 33 to D2 (25%), and 73 to D3 (55.3%). HBsAg subtypes were deduced from the sequence by identifying amino acids at positions 122, 160, 127, 159 and 140. All of the genotype A isolates displayed a sequence characteristic for adw2 HBsAg subtype. Among genotype D isolates, four HBsAg subtypes were identified: ayw1, ayw2, ayw3 and ayw4, where ayw2 was predominant (56.8%).

The genotypic resistance to lamivudine was confirmed in 84 (54.5%) of 154 patients. Among investigated isolates, three primary resistance-associated mutations were found, 2 within the YMDD motif (aa203-206) of the RT region (rtM204V and rtM204I) and one outside the YMDD motif – rtA181T. Most isolates (96.4%) displayed primary resistance-associated mutations in the YMDD motif, while in three (3.6%) isolates resistance was the consequence of non-YMDD mutation. The dominant primary resistance-associated mutation was rtM204V. Compensatory mutations were frequent since at least one was found in 69% of isolates, and they were located

on two positions, rtV173L and rtL180M. The frequencies of discovered resistance-associated mutations are shown in Fig. 1.

Combinations of primary and compensatory mutations, i.e. mutational patterns, were also investigated. Seven patterns were discovered in this study group. Three patterns comprised 3 mutations; another 3 patterns comprised a combination of one primary and one compensatory mutation, while 2 patterns consisted of a single primary mutation. The predominant pattern was rtL180M+rtM204V found in 41.6% of all resistant isolates, followed by a pattern comprising single rtM204I found in 27.4%. The frequencies of lamivudine-resistant mutational patterns among the investigated isolates are shown in Fig.2.

HBsAg mutations associated with diagnostic problems and evasion of HBIg therapy and vaccine-induced immunity were discovered in 45(34.1%) of 154 tested isolates. The discovered HBsAg mutations included single base substitutions at 17 amino acid residues: 100, 101, 110, 120, 126, 128, 129, 130, 131,133, 134, 140, 143, 144, 145, 164 and 166, whereas in one isolate a 6-nt insertion was found that added three codons to HBsAg: 115-117.

The genotypic resistance was not statistically associated with any of the discovered genotypes, subgenotypes or HBsAg subtypes. Genotype D was significantly associated with a resistant pattern that included the presence of a single mutation rtM204I without compensatory mutations (ρ=0.183, P=0.03), since this pattern was exclusively found in genotype D. In addition, most isolates that displayed clinically relevant HBsAg mutations belonged to D genotype, thus making the correlation with this genotype highly significant (ρ=0.239, P=0.006).

The presence of resistance-associated mutations was further correlated to older age of patients (ρ=0.289, P=0.001) and presence of clinically relevant HBsAg mutations (ρ=0.239, P=0.006). The correlation of resistance-associated mutations with higher values of viral load was at statistical borderline (ρ=0.242, P=0.06).

DISCUSSION

Nucleos(t)ide analogs revolutionized the treatment of chronic HBV infection because of sustained suppression of viral replication and remission of liver disease. However, long-term NA therapy exposes patients to the risk of a selection of drug-resistant mutants (Papatheodoridis et al., 2005, Locarnini and Mason, 2006). Because of the spontaneous variability of the HBV genome, NA pressure could select for viral quasispecies that exhibit the best replication capacity in the presence of an antiviral agent. Genotypic antiviral resistance designates the presence of a unique nucleotide and the corresponding deduced amino acid mutations in the drug target gene that have been previously demonstrated to be associated with antiviral resistance (Lok et al., 2007a).

Lamivudine monotherapy is responsible for fastest drug-resistance selection among NA. According to Lai et al. (2003), 14%-32% of patients become resistant to the drug each year after treatment was initiated, and more than 80% are resistant after 48 months of treatment. Two previous studies concluded that the rate of LMV resistance was approximately 20% per annum and that after 5 years it reached 69% (Benhamou et al., 1999, Liaw et al., 2000). This is in accordance with the present study, which demonstrated a high rate (54.5%) of confirmed genotypic resistance in patients treated for at least two years. Because of the high probability of HBV resistance, LAM monotherapy is not currently considered an optimal first-line long-term treatment for chronic hepatitis B (Lok and McMahon, 2007b). However, it is still widely used, mostly because of its low cost.

The HBV genotype, subgenotype and HBsAg subtype distribution among investigated isolates was in accordance with previously published data for Serbian patients (Lazarević et al. 2010). Accordance was also observed for the frequency of clinically relevant HBsAg mutations that were present in approximately 1/3 of patients on long-term NA therapy in the previous and present study.

The association of specific HBV genotype and HBsAg subtype with the development of antiviral drug resistance was first suggested by Zollner et al. (2001). This study reported that an adw HBsAg subtype had a 20-fold higher risk of lamivudine resistance than ayw. Later, Verschuere et al. (2005) reported that the rate of resistance after 1 year of lamivudine therapy is higher in patients with genotype A infection than in patients with genotype D infection, but YMDD variants appear earlier in genotype D. However, in the present study the occurrence of resistance was similar in genotypes D (65.8%) and A (50%) and no statistical difference was found. A few other studies (Buti et al., 2002, Akuta et al., 2003, Moskovitz et al., 2005) also reported that the emergence of lamivudine resistance was comparable between genotypes A and D. Finally, a more recent meta-analysis consistently found no significant association between HBV genotype and response to nucleos(t)ide analogs (Wiegand et al., 2008).

The differences between genotypes A and D with respect to mutational pattern during the development of lamivudine resistance were also suggested (Zollner et al., 2004). In this study, all seven mutational patterns were present in genotype D isolates, while genotype A isolates displayed only three, where rtL180M+rtM204V was dominant and also more frequent than in genotype D (38.9% in A vs. 24.6% in D). This is consistent with the report by Zollner et al. (2004) that genotype A had a 6-fold higher probability of selecting a valine substitution compared to genotype D. Also consistent with previous reports, the findings of this study conclude that mutation rtM204I was more frequent in genotype D and that the pattern including single rtM204I, without compensatory mutations, was found exclusively in genotype D isolates.

Compensatory mutation rtL180M alone was found to confer modest resistance to lamivudine. However, mutations in the B domain of RT (rtL180V and or rtV173L) were found to restore the replication capacity nearly to that of the wild-type virus (Delaney et al. 2003). This could explain the frequently observed combined mutation patterns of primary and compensatory mutations in chronically infected patients. Not surprisingly and consistent with other reports (Tacke et al., 2004, Thompson et al., 2007), the two most prevalent mutation patterns in this study were L180M+rtM204V followed by unassociated rtM204I.

Despite being outside the YMDD motif, the rtA181T mutation may alter the position of codon rtM204, resulting in an allosteric change of the catalytic site, and it may also alter the positioning of LMV relative to rtM204 (Bartholomeusz and Locarnini 2006). This may potentially lead to a mechanism for discrimination between the natural substrate and the nucleoside analog, resulting in resistance. Since it is often occurs in the absence of rtM204V/I, it is considered a primary resistance mutation (Yeh et al., 2000). This mutation at codon rtA181T was not so frequent in the investigated group but since it is also selected during adefovir treatment, it could be responsible for cross-resistance between these two NAs.

Lamivudine-associated resistance mutations in the P gene are responsible for corresponding changes in the S gene and HBsAg, and therefore can affect antigenicity. Thus, all isolates that display resistance mutations are potential vaccine-escape mutants (Torresi et al., 2002). Besides, HBsAg mutations that were independent of resistance-associated polymerase mutations were more often found in treated patients and it is believed that some of them are triggered by LAM. The prevalence of clinically relevant HBsAg mutations was high in the present study (34.1%) and their presence was significantly correlated with genotype D, which can be explained by the established higher variability of this genotype (De Maddalena et al., 2007).

In conclusion, the genotypic resistance to lamivudine monotherapy was found in more than half of investigated patients on long-term treatment. It was shown that the older age of patients and higher viral load were predictive factors for the occurrence of resistance. High prevalence of resistance supports the use of genotypic testing in monitoring patients on lamivudine therapy and selecting those who

would benefit from therapy with newly developed nucleos(t)ide analogs.

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