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Mutations in *Pol* gene of hepatitis B virus in patients with chronic hepatitis B before and after therapy with nucleoside/nucleotide analogues

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Summary. – Chronic hepatitis B (CHB) is one of the most common infections worldwide. Currently approved treatments of CHB include nucleoside/nucleotide analogues (NAs). However, long-term NA therapy is associated with accumulation of resistant mutations within the hepatitis B virus (HBV) polymerase gene. The incidence of naturally occurring HBV mutations leading to primary antiviral resistance has not been fully elucidated yet. The objective of present study was to detect the frequency of mutations within the HBV polymerase gene in 263 patients naïve to nucleoside/nucleotide analogues. Prevalence of HBV *Pol* gene mutations secondary to NA treatment in patients without pre-existing antiviral resistance mutations was also examined. Retrospective analysis showed that HBV *Pol* gene mutations were present in 7 out of 263 patients prior to the treatment. Mutations observed in NA-naïve CHB patients were associated only with resistance to lamivudine and adefovir. Compensatory mutations were observed as well. In the course of antiviral treatment, HBV *Pol* gene mutations were identified in 65 out of the remaining 256 CHB patients (25.39%), while no mutations of any type were detected in 160 patients (62.5%). The profiles of detected mutations were comparable to those observed in other studies that focused on the analysis of clinically relevant NA-resistant mutations. In conclusion, we found out that antiviral resistance mutations may pre-exist in the overall viral population present in untreated patients, although the incidence of HBV *Pol* gene mutations in NA-naïve CHB patients was low and reached only up to 2.66%. However, possible circulation and transmission of NAs-resistant HBV mutants in human population should be taken into account.

Keywords: chronic hepatitis B; drug resistance; HBV polymerase gene; nucleotide analogues

Introduction

Hepatitis B virus (HBV, the family *Hepadnaviridae*) is a small DNA virus capable of infecting only humans and certain primates (Liang, 2009; De Clerq, 2010; Seeger and Mason, 2000). Although its active replication occurs primarily in liver cells, the virus was also demonstrated to be present outside hepatocytes, e.g. within the cells of lymphoid tissue, bile duct and kidney (Seeger and Mason, 2000; Zoulim and Locarnini, 2009). Since HBV reverse transcriptase has no proofreading activity, it promotes natural genetic variability of the virus (mutation ratio is about 10^{-7} to 1 nucleotide substitution per day) and increases the frequency of mutations, thus reducing sensitivity to antiviral drugs (Seeger and Mason, 2000). Due to the high rates of HBV mutations and replication means,

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Abbreviations: CHB = hronic hepatitis B; HBV = hepatitis B virus; NA = nucleotide analogue; HBsAg = hepatitis B antigen

the serum of CHB patients initially contains a heterogeneous viral population consisting of several HBV variants differing at least by single point mutations (Nafa, 2000). Inability to repair replication errors leads to accumulation of DNA sequence mutations of the individual variants. Most important factors responsible for the development of resistance to nucleoside/nucleotide analogues include high pre-treatment levels of HBV DNA and alanine aminotransferase (ALT) as well as high body mass index values (Zoulim, 2011; Locarnini, 2003; Stuyver, 2001; Locarnini and Mason, 2006). Drug resistance mutations are adaptive due to high genetic barrier and pharmacodynamic properties of drugs as well as higher replication rates of mutant HBV (Durendel, 2010).

HBV DNA polymerase (HBV *Pol*) gene consists of four domains: the terminal domain acting as primer for reverse transcriptase, a spacer region of unknown function; the reverse transcriptase/polymerase (RT/*Pol* domain) and ribonuclease H *Pol* domain which is divided into five subdomains labeled A to E (Locarnini, 2008). Individual genotypes differ in the length of the HBV *Pol* gene mainly due to mutations in the spacer region. No differences were observed in the length of the RT domain (Niesters, 2010; Yildiz, 2011). Since the HBV genome is organized in overlapping reading frames, the polymerase gene overlaps the genes encoding the viral envelope. Consequently, mutations within the HBV *Pol* gene can produce changes in the overlapping envelope genes, particularly the *S* gene (Yildiz, 2011; Arrese, 2011). *In vitro* studies have shown that HBV variants with mutations within the overlapping regions of genes encoding polymerase and envelope proteins are characterized by slower replication rates and smaller infectivity (Nafa, 2000; Arrese, 2011). At the same time, some HBV mutants became invisible to antibodies in blood due to mutations in surface antigens. This may contribute to the development of infections in people vaccinated against hepatitis B (Nafa, 2000; Locarnini, 2008; Arrese, 2011). *In vitro* studies confirmed a reduced ability of anti-HBs antibodies to bind HBV variants with mutations rtV173L+rtM204V+rtL180M within the polymerase gene and sE164D+sI195M within the HBsAg-encoding gene (Nafa, 2000; Arrese, 2011; Zöllner, 2005). Antiviral resistance mutations may pre-exist in the overall population of untreated patients infected with HBV. The incidence of naturally occurring HBV mutations leading to primary antiviral resistance has not been fully elucidated yet.

The objective of present study was to detect the frequency of mutations within the HBV *Pol* gene in patients naïve to nucleoside/nucleotide analogues. Prevalence of HBV *Pol* gene mutations secondary to NA treatment in patients without pre-existing antiviral resistance mutations was also examined.

Materials and Methods

Patients. The study population consisted of 263 NAs-naïve patients recruited between October 2009 and April 2012 at the Department of Infectious Diseases, University of Medical Sciences, Poznan, Poland. Study population consisted of 90 women and 173 men at the mean age of 37.1 years. All patients were diagnosed with CHB and were positive for HBsAg and anti-HBe, with HBV DNA levels of >1000 IU/ml. According to the guidelines of the National Health Fund in Poland, patients enrolled between year 2009 and April 2011 were treated with lamivudine (Zeffix) at 100 mg/day as a the first-line CHB treatment. Resistance to the NA and the lack of response to previous treatment resulted in the use of other NA – adefovir (Hepsera) 10 mg/day and/or entecavir (Baraclude) 1 mg/day and/or tenofovir (Viread) 200 mg/day. Starting from April 2011, peginterferon alpha-2a (Pegasys) was used in the first-line treatment of previously untreated CHB patients at the dose of 180 μg once a week. If no elimination of HBV DNA was observed, entecavir (Baraclude) 1 mg/day and/or tenofovir (Viread) 200 mg/day was implemented as second-line therapy.

Methods. NucleoSpin Blood Kit (Machery-Nagel) was used for isolation of HBV DNA from the serum. Isolation procedure was carried out according to the manufacturer's instructions. INNO-LiPA HBV DR v2/v3 (Innogenetics NV, Belgium) line probe assay was used for detection of resistance-carrying mutations within the HBV polymerase gene. The study was conducted using HBV DNA samples isolated at baseline (before treatment) and after 12, 24, and 48 weeks of treatment, as well as whenever resistance to NAs was suspected.

Results

Retrospective analysis showed that HBV *Pol* gene mutations were present in 7 out of 263 patients prior to the treatment. Following mutations were detected in NA-naïve patients: rtL80V (2 patients), rtL80I (1 patient), rtL180M/A181T/V (2 patients), rtM204V (1 patient) and rtM204I (1 patient). In four patients within this group, additional mutations appeared in the course of NA therapy, while in the remaining three patients mutations remained the same as those observed before treatment (Fig. 1). To date, no elimination of HBV DNA could be achieved in any of these seven patients.

Only the remaining 256 CHB patients in whom no pre-treatment mutations were detected were included in further analyses. In the course of antiviral treatment, HBV *Pol* gene mutations were identified in 65 out of 256 CHB patients (25.39%) while no mutations of any type were detected in 160 patients (62.5%). In the remaining 31 patients (12.11%), HBV DNA was eliminated from the serum, making determination of potential HBV *Pol* gene mutations impossible.

Genotyping of the HBV *Pol* gene allowed us to determine the profile of mutation occurrence. Table 1 lists the detected mutations according to their frequency in individual patients in the course of NA treatment. Mutations resistant only to lamivudine were detected in 19 (29.23%) out of 65 patients. Three (4.62%) patients presented selective mutations associated with resistance to adefovir while isolated compensatory mutations were detected in another two (3.08%) patients. In the remaining patients, complex HBV *Pol* gene mutations, including mutations determining resistance to lamivudine and/or adefovir as well as compensatory mutations, were detected. In these patients, the initially isolated mutations became more and more complex during treatment. No mutations associated with resistance to entecavir were detected. This might be due to the short, 6-month duration of entecavir use in our CHB patients.

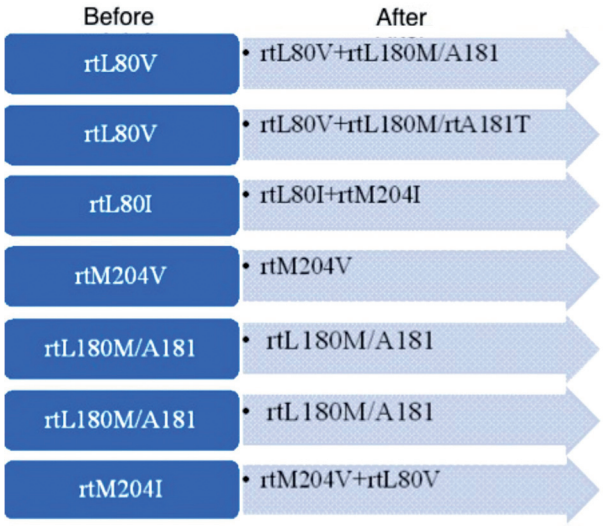

Fig. 1
HBV *Pol* gene mutations in CHB patients before and after NAs therapy
Abbreviation of mutations: as an example – in rtL80V *rt* stands for reverse transcriptase and amino acid L at position 80 is replaced with amino acid V; in rtL180M/A181T/V *rt* stands for reverse transcriptase and amino acid L at position 180 is replaced with amino acid M and amino acid A at position 181 is replaced with amino acid T or V.

Discussion

HBV variants may develop in CHB patients either as a result of positive selection by patients' own immune response, or during the treatment with NAs. Rates of primary NAs resistance in newly diagnosed HBV patients have been

Table 1. The incidence of mutations in HBV *Pol* gene in CHB patients following the NAs therapy

Mutation	No. (%) of patients with mutations
rtL180M/A181+rtM204V	15 (23.1%)
rtM204I	7 (10.77%)
rtM204V	7 (10.77%)
rtL180M/A181	5 (7.69%)
rtL80V+rtL80I+rtM204I	4 (6.15%)
L180/rtA181T	3 (4.62%)
rtL80V+rtL180M/A181+rtM204V+rtM204I	3 (4.62%)
rtL180/rtA181T+rtM204I	2 (3.08%)
rtL80V	2 (3.08%)
rtL80V+rtM204I	2 (3.08%)
rtL80V+rtV/G173L+rtL180M/A181+rtM204I	1 (1.54%)
rtL80V+L180/A181	1 (1.54%)
rtL80V+rtL180M/A181+rtM204V	1 (1.54%)
rtL80V+rtL180M/rtA181T	1 (1.54%)
rtL80V+rtM204V+rtM204I	1 (1.54%)
rtL80V+rtL80I+rtL180M/rtA181T	1 (1.54%)
rtL80I+rtM204I	1 (1.54%)
rtL80I+rtL180M/A181+rtM204V	1 (1.54%)
rtV/G173L+rtM204I	1 (1.54%)
rtV/G173L+rtL180M/A181+rtM204V+rtN236T	1 (1.54%)
rtV/G173L+rtL180M/A181+rtM204V	1 (1.54%)
L180/A181+rtM204V	1 (1.54%)
rtL80V+rtL180M/rtA181T+rtM204V+rtM204I	1 (1.54%)
rtM204V+rtM204I	1 (1.54%)
rtL80V+rtL80I+rtL180M/A181+rtM204V	1 (1.54%)

reported only occasionally (Mirandola, 2011; Cuestas, 2010). Naturally occurring HBV variants with primary antiviral resistance were rarely observed. In a group of Argentine and Italian patients, the frequency of primary antiviral resistance did not exceed 5% (Mirandola, 2011; Cuestas, 2010). Naturally occurring HBV variants were also tested in HIV/HBV-coinfected patients in Spain. Also in this case, the frequency of primary antiviral resistance was low (5.5%) (Tuma, 2011). In our paper, we found out that antiviral resistance mutations may pre-exist in the overall viral population present in untreated patients, although the incidence of HBV *Pol* gene mutations in NA-naïve CHB patients was low and reached only up to 2.66%. In line with the findings from other research groups, only mutations associated with resistance to lamivudine and adefovir, as well as compensatory mutations were observed in treatment-naïve patients in our study. As an exception, the Italian research group has additionally identified the rtM250L/V mutation typical for entecavir resistance (Mirandola, 2011). This might be due to the fact that the widespread use of lamivudine as oral therapy for chronic HBV infections was favored selection and thanks to worldwide circulation of resistant HBV strains. Transmission of such resistant HBV variants can be rapid, particularly within the population of HIV patients, who are more exposed to lamivudine as part of antiretroviral therapy.

We have traced the original mutations of the HBV *Pol* gene in the seven NA-naïve CHB patients over the course of treatment (Fig. 1). Only one out of four patients with primary resistance to lamivudine developed the compensatory mutation rtL80V. The remaining three of these four patients have retained their status of HBV *Pol* gene mutations. In another three patients with primary compensatory mutations, NA resistance mutations were induced over the course of treatment, including mutations resistant to lamivudine as well as adefovir.

Prevalence of HBV *Pol* gene mutations secondary to NA treatment in patients without pre-existing antiviral resistance mutations was also examined. The profile of HBV *Pol* gene mutations as detected in our study was similar to the results obtained by other groups (Cuestas, 2010; Tuma, 2011; Delaney, 2003). Compensatory mutations were identified in 24 patients. The most frequent mutation was rtL80V/I (observed in 21 patients). The rtV173L substitution was identified in only 3 patients and was associated with mutation rtM204V/I. Similar drug resistance profile has also been reported in other studies (Zoulim and Locarnini, 2009; Arreses; 2011; Zöllner, 2005). Delaney *et al.* analyzed the prevalence of rtV173L mutation and its relation to rtL180M and rtM204V/I (Delaney, 2003). As shown by the study, the rtV173L mutation is always accompanied by the combined mutations rtL180M+rtM204V. In the same paper, the effect of the rtV173L mutation on the efficiency of virus replication *in vitro* was also examined. It was demonstrated that rtV173L mutation plays a compensatory role in lamivudine-resistant variants of HBV, increasing the efficiency of replication in both wild-type and mutated HBV variants.

Changes within the HBV *Pol* gene were found in 65 out of 256 patients without pre-existing antiviral resistance mutations. Most frequently identified was lamivudine-resistant mutation rtM204V, either as an isolated mutation or in combination with other mutations. In a majority of patients, mutation at codon rt204 was accompanied by rtL180M within the B subdomain of the *Pol* gene. Although the position rt180 is located outside the catalytic center of the enzyme, the rtL180M mutation is also associated with the development of resistance to lamivudine. A combination of rtL180M+rtM204V/I mutations is often detected in HBV DNA obtained from patients after failure of lamivudine therapy, as already been reported in a number of studies (De Clercq, 2010; Zoulim and Locarnini, 2009; Arrese, 2011).

The treatment of chronic hepatitis B remains limited to monotherapy with either peginterferon-alpha or one of five different NAs. Viral suppression can be achieved in approximately 95% of CHB patients treated with new-generation NAs. However, the rate of HBeAg seroconversion ranges only from 20% to 30% after a follow-up of 5 years (Zoulim 2012). Accumulation of drug-resistant mutations within the polymerase gene of HBV is an important therapeutic challenge. The prevalence of variants with reduced susceptibility to lamivudine and other NAs generates the clinical need of finding new drugs with mechanisms of action different from that of polymerase HBV inhibitors.

To conclude, in view of the obtained results, showing that mutations within the HBV *Pol* gene associated with resistance to currently available NAs may pre-exist in patients who never received these drugs. One should be mindful of the possibility of circulation and transmission of HBV mutants with resistance to NAs in human population.

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