

CIRCULATION OF HEPATITIS B VIRUS VARIANTS CARRYING MUTATIONS IN POLYMERASE GENE AMONG HBV-INFECTED AND HBV/HIV COINFECTED PATIENTS

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The aim of the investigation was to study the prevalence of hepatitis B virus (HBV) variants with polymerase gene mutations among HBV-infected and HBV/HIV-coinfected patients previously received no nucleotide/nucleoside analogues therapy.

Materials and Methods. We studied the blood serum samples of 459 patients with HBV-monoinfection and 590 HIV-infected patients (a group with a high risk of HBV infection) who did not receive nucleotide/nucleoside analogues therapy. HBV DNA was determined by PCR method with primers to conservative part of overlapping S and P genes coding the surface protein and DNA-polymerase of HBV, respectively. HBV polymerase gene was sequenced for 241 isolates from HBV-infected patients, and 30 — from patients with HBV/HIV-coinfection. Phylogenetic analysis of HBV sequences was performed using Mega 4.0. program.

Results. The viral genome in 7 of 241 patients (2.9%) with HBV-monoinfection was found to have significant amino-acid replacements: in 2 patients (0.8%) — A181S mutation associated with adefovir-resistance, in 4 (1.7%) — T184I related to HBV entecavir-resistance, and in 1 (0.4%) — L199W associated with telbivudine-resistance development. Among 30 examined patients with HBV/HIV-coinfection, 1 patient (3.3%) was found to have significant amino-acid replacement in HBV polymerase gene (in YMDD-motif — M204I) associated with the development of resistance to lamivudine, entecavir, telbivudine and tenofovir. Overall detection rate of primary drug resistance of HBV was 6.2% among HBV-infected and 10% — among HBV/HIV-coinfected patients.

Conclusion. The obtained data on the prevalence of HBV primary drug resistance indicate the need for screening of patients with HBV/HIV-coinfection before starting the antiviral therapy. The patients are to be screened on mutations associated with resistance to the main nucleotide/nucleoside analogues used in hepatitis B therapy.

Key words: viral hepatitis B; nucleotide/nucleoside analogues; drug resistance.

The importance and complexity of hepatitis B virus (HBV) treatment is caused by its ubiquitous distribution among different population groups, a variety of clinical forms and outcomes (including liver cirrhosis and hepatocellular carcinoma), stably high morbidity rates [1].

Persistence of viral genome in infected hepatocytes and high rate of spontaneous mutations form the basis of selection of mutations in hepatitis B virus, leading to the formation of drug resistance to reverse transcriptase inhibitors. It is established that mutations in the HBV genome

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associated with drug resistance may occur spontaneously before starting antiviral therapy using reverse transcriptase inhibitors [2]. This phenomenon is called HBV primary drug-resistance [3].

Early detection of HBV variants with mutations in polymerase gene (P gene) associated with the development of drug resistance has important clinical significance first of all to prevent exacerbation of the disease. Early detection of such HBV variants is very important for patients with a high risk of HBV progression, especially HBV / HIV-coinfected, both before treatment with nucleos(t)ide analogues and in the course of treatment, when the viral load may be very low and/or when fewer HBV mutations are present in the total virus population [4].

There is little information on hepatitis B virus variants carrying mutations in polymerase gene of HBV-infected and HBV / HIV-coinfected patients, what proves the importance of the study of the circulation of drug-resistant HBV variants in patients that previously were not treated against HBV.

The aim of the investigation was to study the prevalence of hepatitis B virus (HBV) variants with polymerase gene mutations among HBV-infected and HBV/HIV-coinfected patients that previously received no nucleotide/nucleoside analogues therapy.

Materials and Methods. We studied the blood serum samples of 459 patients with HBV-monoinfection and 590 HIV-infected patients (a group with a high risk of HBV infection) who did not receive nucleotide/nucleoside analogue therapy, from regions of the Russian Federation with different HBV morbidity rates, i.e. with varying degrees of HBV circulation intensity among population (Table 1).

Written informed consent was obtained from all patients.

We detected serological markers of HBV infection (HBsAg and anti-HBc) using the following enzyme immunoassay test systems in accordance with the manufacturer's instructions: "DS-EIA-HBsAg-0.01", "DS-EIA-HBsAg-0.01-confirmatory" (NPO "Diagnostic Systems", Nizhny Novgorod, Russia) in order to confirm HBsAg detection by neutralization reaction and "DS-anti-HBc" (NPO "Diagnostic systems", Russia).

We detected HBV DNA in all anti-HBc and HBsAg-positive samples of blood serum using polymerase chain reaction (PCR). Extraction of nucleic acids from blood serum samples was performed by phenol-chloroform extraction using a kit for extraction of DNA/RNA from blood serum or blood plasma (Research and Production Company Lytech, LLC). HBV DNA was determined by PCR method with primers to conservative part of overlapping S and P genes encoding the surface protein and DNA-polymerase, respectively. The detection sensitivity of HBV DNA in this reaction was not less than 100 copies/ml based on the testing results of a series of limiting dilutions of samples with known HBV DNA concentrations. The conditions for both PCR rounds were as follows: 94°C — 2 min, then 35 cycles: denaturation at 94°C — 45 s, annealing at 55°C — 45 s and chain extension at 72°C — 1 min 30 s. The PCR product of 713 base pair was determined by agarose gel electrophoresis (1.5%) in tris-borate buffer (TBE).

For samples positive for HBV DNA by PCR we determined the sequence of reverse transcriptase domain of HBV DNA polymerase in order to identify possible

Table 1

The examined patients with HBV

Russian Federation regions	Number of patients (n=459)	HBV circulation intensity (endemicity)
The Sakha Republic (Yakutia)	58	High
The Republic of Tuva	137	High
Saratov region	49	Medium
Moscow region	100	Low
Tambov region	115	Low

mutations in this part. The amplification product with primers to conservative part of overlapping S and P genes of 713 base pair were excised from the gel and extracted from the agarose using QIAquick Gel Extraction kit (QIAGEN, Germany). Sequencing was performed using GenomeLab Methods Development kit (Beckman Coulter, England) in analysis system CEQ 8800 (Beckman Coulter, England). The received sequences were compared with the reference sequences deposited in the GenBank database (USA), which corresponded to "wild-type" HBV and to mutation forms with documented drug resistance to five nucleot(s)ide analogues, currently used for the treatment of HBV infection: lamivudine, adefovir, entecavir, tenofovir, telbivudine. Phylogenetic analysis of HBV sequences was performed using Mega 4.0.

Results and Discussion. During examination of 459 patients with HBV infection, HBV DNA was detected in 241 patients with the presence of anti-HBc and HBsAg. The viral genome in 7 of 241 patients (2.9%) patients with HBV-monoinfection was found to have significant amino-acid replacements (mutations are described in the literature as causing development of drug resistance to therapy with nucleos(t)ide analogues) and in 8 patients (3.3 %) — potentially significant (replacements in the same significant positions of the polymerase gene, but not yet described in the literature). In none of the cases in this group of patients were detected significant amino acid replacements in the YMDD-motif of HBV being the most common mutations and associated with the development of resistance to a wide range of reverse transcriptase inhibitors (lamivudine, entecavir, telbivudine and tenofovir). However, the analysis of nucleotide sequences of HBV polymerase gene among HBV-monoinfected patients revealed mutations associated with the development of primary drug resistance to other nucleos(t)ide analogues. The range of amino acid replacements and their detection rate are shown in Table 2.

In addition to these amino acid replacements HBV-monoinfected patients were found to have also potentially significant mutations. HBV genome of one HBV-monoinfected patient (0.4%) had threonine replaced with proline at position 184 (T184P). However, according to the literature, threonine at this position of HBV P-gene is replaced with isoleucine, leucine, serine and adenine that lead to the occurrence of resistance to entecavir therapy [5, 6].

Table 2
Significant amino acid replacements
in HBV polymerase gene (n=241)

The results of the revealed mutations in HBV polymerase gene		Pharmaceuticals
Amino acid replacements	n/%	
A181S	2/0.8	Adefovir
T184I	4/1.7	Entecavir
L199W	1/0.4	Telbivudine

HBV genome in 3 of 241 patients (1.2%) with HBV mono-infection revealed a potentially significant replacement of methionine by leucine at position 204 localized in YMDD-motif of the polymerase gene, which is crucial for the development of resistance to nucleos(t)ide analogue therapy. However, the literature describes other replacements at position 204 (M204I/V/S) associated with the development of resistance to lamivudine, entecavir, telbivudine and tenofovir [7, 8].

HBV polymerase gene has revealed three potentially significant amino acid replacements: the 1st patient (0.4%) had replacement of serine by phenylalanine at position 213 (S213F), the 2nd patient (0.4%) — replacement of leucine by serine at position 179 (L179S) and the 3rd patient (0.4%) — replacement of leucine by proline at position 82 (L82P).

The literature [9, 10] describes other amino acid replacements at positions 82, 213 and 179 (S213T, L179P, L82M) associated with the development of drug resistance to lamivudine. We have identified potentially significant mutations, i.e. amino acid replacements in those positions in which, according to the literature, mutations lead to the development of drug resistance, however the amino acid residues being replaced in the mentioned cases differed from the cases marked by other authors.

Overall detection rate of primary drug resistance of HBV to the nucleos(t)ide analogue therapy was 6.2%.

Having examined 590 patients with HIV infection, we detected HBV DNA in 30 people. Among them, 1 patient (3.3%) was found to have a significant amino acid replacement in HBV polymerase gene — a mutation in the YMDD-motif of HBV polymerase gene — M204I associated with the development of resistance to lamivudine, entecavir, telbivudine and tenofovir.

In this group of HBV/HIV-coinfected patients HBV polymerase gene was found to have also potentially significant mutations (6.7%, 2/30): L179S and V214Y. One of thirty patients (3.3%) had leucine replaced with serine at position 179 (L179S). M.W. Li in collaboration with [11] described the replacement of leucine by proline at position 179 (L179P) associated with the development of drug resistance to treatment of chronic hepatitis B virus by lamivudine. HBV polymerase gene in one HBV/HIV-coinfected patient had valine replaced with tyrosine at position 214 (V214Y). In this position of the HBV polymerase gene, V. Soriano [12] described the replacement of valine by alanine (V214A) associated with the development of drug resistance to adefovir. The mutations that we detected

in the course of our study may be new and crucial for the development of drug resistance. However, this assumption requires a detailed examination of the phenotypic manifestation of infection with HBV variants carrying these amino acid changes in the genome.

Taking into account all significant and potentially significant mutations identified during our study in the HBV polymerase gene associated with the development of resistance, overall detection rate of primary drug resistance in group of HBV/HIV-coinfected patients was 10.0% (3/30).

Thus, the received results demonstrated circulation of HBV variants carrying mutations in the polymerase gene among HBV infected and HBV/HIV-coinfected patients. A significant number of people initially have resistance to one antiviral drug or another.

Conclusion. In order to reduce the incidence of drug resistance in HBV-infected and HBV/HIV-coinfected patients, they should be monitored with the use of highly sensitive and highly specific methods for the detection of mutant variants of HBV prior to starting treatment with reverse transcriptase inhibitors for planning of optimal schemes of drug administration and its control during application.

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