An initial assessment of correlations between host- and virus-related factors affecting analogues antiviral therapy in HBV chronically infected patients

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Source of support: Ministry of Science and Higher Education, grant no. N N302183639 and Medical University of Gdansk, grant no. ST79

Background: Success in treating hepatitis B virus (HBV) infection with nucleoside analogues drugs is limited by the emergence of drug-resistant viral strains upon prolonged therapy. In addition to mutation patterns in the viral polymerase gene, host factors are assumed to contribute to failure of treatment in chronic HBV infections. The aim of this study was to analyze the correlation between efficacy of antiviral therapy and the prevalence of HBV pretreatment drug-resistant variants. We also analyzed the role of heterogeneity in the promoter region of the IL-10 on the HBV pol/s gene polymorphisms and efficacy of analogues-driven therapy.

Material/Methods: HBV DNA was extracted from 54 serum samples from chronic hepatitis B (CHB) patients. Drug-resistance mutations were analyzed using MALDI-TOF mass spectrometry technology (MALDI-TOF MS) and Multi-temperature single-strand conformation polymorphism (MSSCP). IL-10 gene promoter region polymorphisms at positions -1082, -819, and -592 were determined in allele-specific PCR reactions (AS-PCR).

Results: Drug-resistance mutations were detected in 74% of naïve and 93% of experienced patients, but the effect of pre-existence of drug-resistant HBV variants on antiviral therapy was not statistically significant (p=0.86). The role of polymorphisms at positions -1082 (p=0.88), -819 (p=0.26), and -592 (p=0.26) of IL-10 promoter region polymorphisms was excluded from the response-predicting factors. The main host factors predicting successful response to antiviral therapy were female sex (p=0.007) and young age (p=0.013).

Conclusions: The presence of drug-resistant HBV variants in baseline is not a viral predictor of good response to nucleoside/nucleotide analogues therapy. Only low HBV viral load predicted positive response to antiviral therapy. The ideal candidate for antiviral therapy is an immunocompetent, young female with low HBV viral load and elevated ALT activity.

MeSH Keywords: Polymorphism, Genetic • Drug Resistance • Interleukin-10 • Mass Spectrometry • Hepatitis B, Chronic

Full-text PDF: http://www.medscimonit.com/download/index/idArt/889788
Background

Hepatitis B virus (HBV) infection, affecting about 2 billion people worldwide, is still a global health problem. Despite the existence of a highly effective vaccine, 240–350 million chronic HBV carriers could develop chronic hepatitis B (CHB) with high risk of liver cirrhosis and hepatocellular carcinoma (HCC) [1–4]. The primary treatment goal in CHB patients is the prevention of liver disease progression. Life-long suppression of HBV replication seems to be the best antiviral strategy [4–6]. However, the existence of covalently closed circular DNA (cccDNA) in infected hepatocytes probably makes the eradication of HBV infection impossible [7,8].

Nucleotide/nucleoside analogues (NAs) and peg-interferon alpha (PegIFNα2a) are 2 major classes of antiviral drugs approved for CHB treatment. NAs agents (e.g., lamivudine, telbivudine, entecavir, adefovir, and tenofovir) can suppress viral load, but long-term therapy can lead to the selection of drug-resistant HBV variants [4,9]. Recent studies showed that there is a possibility of the pre-existence of natural resistance mutations in treatment-naïve patients [10–12]. Viral quasispecies evolve over time under selective pressure (e.g., antiviral therapy). These variants are more viable and spread more rapidly in the liver than does wild-type HBV [1,6,13].

Early detection of drug-resistant HBV variants is crucial for patients treated with low genetic barrier drugs [14,15]. Several methods are currently available to monitor HBV drug resistance (direct sequencing of PCR products, restriction fragment length polymorphism – RFLP, and mutation-specific real-time PCR) but reverse hybridization – Line Probe Assay is the most popular. Among these methods, MSSCP assay (multi-temperature single-strand conformation polymorphism) and MALDI-TOF MS (matrix-assisted laser desorption ionization time of flight mass spectrometry) are the most sensitive, being able to detect HBV mutants, which constitute only 1% of the viral population [16–19].

Development of HBV mutants is also related to the persistence of cccDNA and host factors affecting immune response [13,20]. Polymorphic sites within the IL-10 gene promoter region at positions –1082, –819, and –592 have been described [21–23]. It was published that certain polymorphisms within the IL-10 gene promoter region are associated with the development of chronic HBV infection and effects of IFN therapy [21,24]. However, the impact of these polymorphisms on the development on drug-resistant HBV strains and response to NAs therapy has not been determined.

The aim of this study was to analyze the correlation between efficacy of antiviral therapy and the prevalence of HBV pretreatment drug-resistant variants. Moreover, the role of heterogeneity in the promoter region of the IL-10 on the HBV pol/s gene polymorphisms and efficacy of analogues-driven therapy was analyzed.

Material and Methods

Study population and sample design

We enrolled into the study 54 consecutive CHB (29 male and 25 female) patients, mean age 48.7±2 yrs, qualified between January 2011 and June 2012 to antiviral therapy at the Department of Infectious Diseases, Medical University of Gdansk. They were qualified and received treatment according to recommendations of the Polish National Health Service (NFZ). These recommendations require the use of peg-IFNα2a or lamivudine (when cytokines are contradicted) as a primary therapy in HBeAg-negative subjects. A pretreatment HBV drug resistance test is required in analogues therapy, and the detection of lamivudine-resistant strains allows use of entecavir, adefovir, or tenofovir. In HBeAg-reactive patients, entecavir or tenofovir is recommended as a first-line therapy.

The majority of subjects received lamivudine – 30/54 (55%), entecavir 16/54 (30%), tenofovir 7/54 (13%), and combined therapy (lamivudine with adefovir) in 1/54 (2%). The therapy effect was assessed 48 weeks after NAs administration, according to the NFZ recommendation. Patients were classified into 2 groups: those with detectable HBV DNA (non-responders) and those with undetectable HBV DNA (responders). Patients receiving peg-IFNα2a; HCV or HIV co-infected individuals were excluded from the study.

Whole blood and serum samples for analyzing HBV viral load, HBV pol/S region, and IL-10 promoter region polymorphisms were collected before antiviral therapy (baseline). Serum samples taken at week 12, 24, and 48 were stored for HBV DNA quantitative testing. At baseline, biochemical and serological tests and liver biopsy assessments were part of routine analyses.

The study protocol was approved by the local ethics committee and informed consent for participation was obtained from all enrolled subjects.

HBV viral load and pol/S region analysis

Whole blood samples were collected into Vacutainer tubes without anticoagulant and then incubated in an upright position for 30–45 min to allow clotting. The clot was then removed by centrifuging at 3500 × g for 15 min and serum was immediately transferred into a clean Eppendorf tube. All samples were stored at –20°C until further analysis. Viral DNA was extracted from
Table 1. PCR thermal cycling parameters.

| Step | 1st PCR | 2nd PCR | HBV genotyping | rs1082 | rs819 | rs592 |
|------|---------|---------|----------------|--------|-------|-------|
| No. of amplification cycles | 35 | 35 | 35 | 33 | 33 | 33 |
| Initial inactivation/denaturation | 95°C/10 min | 95°C/10 min | 95°C/3 min | 95°C/3 min | 95°C/3 min | 95°C/3 min |
| Parameters of amplification | Denaturation | 94°C/30 sec | 94°C/30 sec | 94°C/30 sec | 94°C/30 sec | 94°C/30 sec |
| | Annealing | 56°C/30 sec | 57°C/30 sec | 57°C/30 sec | 57°C/30 sec | 57°C/30 sec |
| | Extension | 72°C/30 sec | 72°C/30 sec | 72°C/60 sec | 72°C/60 sec | 72°C/60 sec |
| Hold | 4°C | 4°C | 4°C | 4°C | 4°C | 4°C |

200 µl of serum samples using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) with a slightly modified manufacturer’s protocol: the incubation time with Proteinase K was 1 h instead of 10 min and the final elution volume was 30 µl. Quality and quantity of the DNA was measured using a spectrophotometer (Nanodrop™ ND-1000, Thermo Scientific, USA). The Roche Cobas Amplicor HBV Monitor Assay (Roche Diagnostics, Pleasanton, USA) was used to quantify the level of HBV DNA in serum (sensitivity 34.36 IU/ml) using the manufacturer’s protocol.

Multi-temperature single-strand conformation polymorphism (MSSCP)

The MSCP assay was done by means of the DNA Pointer System (Biovietis, Poland). To increase the sensitivity of HBV YMDD (rt204) variants detection, nested PCR was performed. The reaction mixture for the first step (1st PCR) contained (25 µl): 2 µl of HBV DNA, 1x chelating buffer, 1.2 mM Mg(OAc)2, 0.2 mM dNTP, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Germany), 0.1 mg/ml of casein, 0.01% (v/v) formamide, and 0.125 µM of primers: 1F and 1R (1F: 5'-GACTCGTGGTGGACTTCTCTC-3', 1R: 5'TGATCCTGTGGCAAAGTTCC-3'). The reaction mixture for the second step (2nd PCR) consisted of (25 µl): 1x chelating buffer, 1.2 mM MgCl2, 0.5 mM dNTP, 1 U of AmpliTaq Gold polymerase, 0.25% (v/v) glycerol, 0.4% (v/v) BSA, 0.125 µM of primers: 2F [17] and 2R (5'TAACAGCGGTATAAAGGGCCT-3'), and 2 µl of the first amplification step product. PCR conditions for each step are shown in Table 1. Positive and negative control was used at each step (HBV DNA External Quality Control, PeliSpy™PRO; AcroMetrix, USA). To determine the detection limit, serial dilutions of Quantification Standard QS HBV RealStar® (Altona Diagnostics, Germany) were used: 104 copies/µl (1718 IU/ml), 105 copies/µl (1718 IU/ml), 106 copies/µl (17.18 IU/ml), 107 copies/µl (1.718 IU/ml), 5 copies/µl (0.859 IU/ml), 2 copies/µl (0.3436 IU/ml), and 1 copy/µl (0.1718 IU/ml).

One µl of denatured PCR products (159 bp) containing HBV mutant and wild-type (WT) sequences at the codons of interest were loaded onto a 11% polyacrylamide gel (29:1 acrylamide: bisacrylamide) with addition of 5% glycerol. The electrophoresis was run under sequentially changed gel temperature (15°C, 10°C, and 5°C for 900 Vxh each) at a constant voltage of 40V in 0.5×TBE. After silver staining, specific band patterns characteristic for each HBV variant were cut out from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen, Germany), and then sequenced.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

The MALDI-TOF MS-based HBV genotyping assay started with amplification of 754 bp DNA fragment containing 12 codons: 80, 169, 173, 180, 181, 184, 194, 202, 204, 233, 236, and 250 [27–35]. The PCR reaction mixture contained (25 µl): 200 nmol/l of each primer [25], 5 µl of HBV DNA, 1x chelating buffer, 1.2 mM Mg(OAc)2, 0.2 mM dNTP and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Germany). Amplification conditions of HBV pol/S region analysis are shown in Table 1. PCR products were then purified from non-incorporated dNTPs by treating with shrimp alkaline phosphatase (SAP) solution (40 min at 37°C and 5 min at 85°C). The iPLEX Gold assay was done in 4 separate primer-extension reactions on the Mass Array genotyping platform (Sequenom Inc., USA) with a standard procedure following the iPLEX kit protocol (Sequenom Inc., USA). To desalt the iPLEX reaction products, a resin kit was used (SpectroCLEAN resin, Sequenom Inc., USA), then cleaned extension products were dispensed onto a 384-element SpectroChip using the Nanodispenser, and mass differences were detected with MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). All data analyses were carried out with TyperAnalyzer Application, version 4 (Sequenom Inc., USA).
IL-10 promoter region polymorphism

Interleukin 10 promoter region polymorphism DNA was extracted from 500 µl of whole blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Germany). DNA was eluted in 50 µl of AE buffer. To determine 3 biallelic polymorphisms at positions –1082, –819, and –592 in the IL-10 gene promoter region, allele-specific PCR reactions (AS-PCR) were performed. Each polymorphism (rs) was assessed in 2 separate reactions. While the common primer was the same for both reactions (rs1082: 5'-AAGCTTCTGTGGCTGGAGTC-3'; rs819: 5'-GGCACATGTTTCCACCTCTTC-3'; rs519: 5'-GGGGTCATGGTGAGCACTAC-3'), 2 different primers were designed for each allele (rs1082: A: 5'-AACACTAAGGCTTCTTTGGTA-3' and G: 5'-AACACTAAGGCTTCTTTGGGTG-3'; rs819: C: 5'-TACCCTTGTACAGGTGATGTACC-3' and T: 5'-TACCCTTGTACAGGTGATGTACT-3'; rs519: C: 5'-TCCAGAGACTGGCTTCCTACAAG-3'). The Human β-globin primers were used as an experimental control (5'-TTGGACCCAGAGGTTCTTTG-3', 5'-GAGCCAGGCCATCACTAAAG-3'). The PCR reaction mixture contained (25 µl): 1× Taq buffer +NH₄SO₄–MgCl₂ (Thermo Scientific, USA), 2 mM MgCl₂ (Thermo Scientific, USA), 0.2 mM dNTPs (Thermo Scientific, USA), 0.4 µM of each primer, 5 µl of BSA (1 mg/µl), 1 U DreamTaq (Thermo Scientific, USA), and ~20 ng/µl DNA. The PCR conditions are shown in Table 1.

Further analysis involved agarose gel (2%) electrophoresis of PCR products (40 min, 100V). Based on the presence or absence of a PCR product, the patient's genotype was determined. For example, if a product was seen in a “G” tube line without a product in an “A” tube line (rs1082), the patient was a GG homozygote. If PCR products were seen in both lines, the patient was a GA heterozygote (Figure 1).

Statistical analysis

Statistical analysis was done using STATISTICA data analysis software, version 8.0 (StatSoft Inc., USA). All statistical data are presented as a mean ± standard error of means (±SE), or median value. Standard error was used because distributions of data were skewed. Analysis of differences between variations was done using nonparametric statistics: Mann-Whitney’s U test, chi-squared test, and Kruskal-Wallis one-way analysis of variance. Relations between variables were estimated by univariate and multivariate logistic regressions. P-value less than 0.05 was considered statistically significant.

Results

The studied group of 54 consecutive CHB patients consisted of 27 naïve and 27 NAs-experienced individuals. The naïve patients had lower prevalence of HBV drug-resistant strains (p=0.019) and were more frequently treated with lamivudine 23/27 (85%) (χ²=20.5, p=0.0001) (Table 2). Entecavir was administrated in 4/27 (15%) subjects. Undetectable HBV DNA at week 24 and at week 48 was similar (p=0.7 and p=0.98, respectively).

Some HBV drug-resistant variants were detected in 20/27 (74%) naïve and 25/27 (93%) NAs-experienced patients in MSSCP assay, confirmed in randomly selected cases by MALDI-TOF MS (Figure 2A, 2B). The HBV drug-resistant variant could affect prescribed drug activity in 13/27 (48%) naïve and 23/27 (85%) NAs-experienced subjects. Commercially available tests (Line Probe Assay – INNOLiPA HBV DR) did not detect drug-resistant strains in naïve patients qualified to NAs therapy [26]. The National Health Service (NFZ) requires pretreatment drug resistance tests in all patients (naïve and experienced) during qualification to NAs therapy.

The efficacy of antiviral therapy in our study did not depend on liver biopsy inflammation activity (p=0.5), fibrosis stage (p=0.99), polymorphisms promoter IL-10 gene (Table 2), or preexistence of HBV drug-resistant variants (p=0.86). The strongest factor predicting the response to antiviral therapy was baseline viral load, which was significantly lower in the responder than the non-responder group (Table 2). The antiviral therapy

Figure 1. An example of results for 7 patients (28, 75, 76, 87, 90, 97) obtained for rs871. Kc and Kt are negative controls for C and T tubes.
Table 2. Demographic and clinical characteristics in responder v/s nonresponder and naïve v/s NAs-experienced CHB patients.

|                                | Analogues antiviral therapy | Analogues antiviral therapy |
|--------------------------------|----------------------------|----------------------------|
|                                | responder (n=28) | nonresponder (n=26) | significance p<0.05 | Naïve (n=27) | NAs experienced (n=27) | significance p<0.05 |
| Gender (male/female)           | 10/18 | 19/7 | 0.0066 | 11/16 | 18/9 | 0.10 |
| Age (years)                    | 43.4±1.98 | 54.4±3.51 | 0.011 | 47.67±3.3 | 49.70±2.59 | 0.62 |
| ALT (IU/l)                     | 136±41 | 76±11 | 0.75 | 120±40 | 95±20 | 0.74 |
| Baseline viral load (kIU/ml)   | 13702±8385 | 57857±15205 | 0.0035 | 33989±12765 | 35935±12814 | 0.98 |
| HBe Ag (reactive/nonreactive)  | 8/20 | 15/11 | 0.033 | 9/18 | 14/14 | 0.25 |
| HBe Ab (reactive/nonreactive)  | 21/7 | 12/14 | 0.032 | 19/8 | 14/13 | 0.25 |
| Baseline HBV drug-resistant variants* (Yes/No) | 19/9 | 17/9 | 0.86 | 13/14 | 23/4 | 0.019 |
| Immunocompromised (Yes/No)     | 10/17 | 8/17 | 0.71 | 8/19 | 10/15 | 0.52 |
| NAs therapy** (naïve/experienced) | 13/15 | 14/12 | 0.60 | NA$ | NA** | NA** |
| Antiviral therapy              | Lamivudine | 16 | 14 | 23 | 7 |
|                                | Entecavir | 6 | 10 | 4 | 12 | 0.0001 |
|                                | Tenofovir | 6 | 1 | 0 | 7 |
|                                | lamivudine + adefovir | 0 | 1 | 0 | 1 |
| Liver biopsy*                   | inflammation grade | 2 | 2 | 1.75 | 2 | 0.70 |
|                                | fibrosis stage | 1.5 | 1.5 | 0.99 | 1.5 | 1.5 | 0.42 |

Data are presented as a mean value ± standard error (SE); * data presented as a median value; * the presence of HBV drug-resistant minor variant before NAs therapy; ** nucleoside/nucleotide analogues therapy; NA$ not applicable.

Figure 2. HBV YMDD variants obtained for 7 patients (A–F) during MSSCP (A) and MALDI-TOF MS analysis (B).
was significantly different in responder and non-responder groups (p=0.03), but the frequency of lamivudine administration was similar in both groups (Table 2).

The highest mean viral load (kIU/ml) was detected in patients receiving entecavir (66,138±20,800). Significantly lower concentration of HBV DNA was measured in lamivudine (19,378±9,379).

Table 3. Distribution of alleles promoter region IL-10 in CHB patients.

| IL-10 polymorphism | Analogues antiviral therapy | Significance p<0.05 |
|--------------------|-----------------------------|--------------------|
|                    | Responder (n=28) | Nonresponder (n=26) | |
| rs1800896 (1082)   |  |  |
| GG                 | 4 | 4 |  |
| AA                 | 3 | 3 | 0.88 |
| GA                 | 21 | 19 |  |
| rs1800871 (819)    |  |  |
| CC                 | 20 | 15 |  |
| TT                 | 0 | 0 | 0.26 |
| CT                 | 8 | 10 |  |
| rs1800872 (592)    |  |  |
| CC                 | 20 | 15 |  |
| AA                 | 2 | 1 | 0.26 |
| CA                 | 7 | 12 |  |
| ATA haplotype IL-10 (present/absent) | 8 | 11 | 0.3 |

Table 4. Univariate and multivariate logistic regression analyses of host and viral predicting NAs therapy response factors in CHB patients.

| Age (year) | Baseline HBV viral load (kIU/ml) | Baseline HBeAg (reactive) | Baseline ALT (IU/l) | Previous NAs* therapy | Baseline drug resistance presence ** | Liver biopsy inflammation activity* | Liver biopsy fibrosis stage* | ATA IL-10 haplotype | Immunocompetent patient |
|------------|---------------------------------|---------------------------|---------------------|------------------------|-------------------------------------|----------------------------------|----------------------------|-----------------------|----------------------|
| 43.4±1.98  | 13.702±8.385                    | 136±41 | 15 | 1.5 | 2 | 2 | 2 | 17 | 0.038 |
| 54.4±3.51  | 57.857±15.205                   | 76±11 | 11 | 1.5 | 2 | 2 | 2 | 17 | 0.038 |
| OR (95% CI) | 0.95 (0.91–0.99)                | 0.29 (0.09–0.90)          | 1.003 (0.998–1.009) | 1.35 (0.45–4.03) | 1.12 (0.36–3.47) | 1.42 (0.60–3.35) | 0.93 (0.46–1.93) | 0.54 (0.18–1.69) | 0.80 (0.25–2.59) |
| p value    | 0.013                           | 0.033                      | 0.22                | 0.85                  | 0.41                               | 0.86                            | 0.29                       | 0.7                  | 0.038 |
| OR (95% CI) | 0.90 (0.83–0.98)                | 0.29 (0.09–0.90)          | 1.01 (0.99–1.02)   | 1.12 (0.36–3.47) | 1.42 (0.60–3.35) | 0.93 (0.46–1.93) | 0.54 (0.18–1.69) | 0.80 (0.25–2.59) |
| p value    | 0.011                           | 0.033                      | 0.178               | 0.85                  | 0.41                               | 0.86                            | 0.29                       | 0.7                  | 0.038 |

Data are presented as a mean value ± standard error (SE); *data presented as a median value; *nucleoside/nucleotide analogues; **the presence of HBV drug-resistant minor variant before NAs therapy.
In our study there were no significant differences in frequency of HBV drug-resistant variants except for previous insufficient drug resistance test during qualification to antiviral therapy. Although IL-10 has been shown to have an important role in chronic inflammation and fibrogenesis, as well as in IFN-α therapy of hepatitis B patients, it cannot become a predictive factor of response to NAs in chronic HBV infection. Moreover, in contrast to IFN therapy, no differential haplotype distribution responsible for IL-10 expression was observed between NAs responders and non-responding patients [21]. This suggests that the association of remaining putative functional single nucleotide polymorphisms (SNPs) (e.g., 2753 A/C and 3575 T/A) in IL-10 and other host genetic factors should be investigated. It is well known that the actions of cytokines may be profoundly conditioned by the presence of other cytokines. Thus, there is a need to identify new predictors for treatment outcome in these patients. New studies of particular combinations of IL-10 SNPs and other host predictors could reveal the relation with susceptibility to nucleos(t)ide analogues and may help in making appropriate treatment decisions [20–23].

In the presented study, female sex and young age were the main host factors predicting successful response to antiviral therapy. The presence of drug-resistant HBV variants at baseline was not a viral predictor of good response to NAs therapy. Only low HBV viral load was useful in identifying the likelihood of response to antiviral therapy. Multivariate analysis connected essential host and viral factors predicting response to NAs drugs, investigated in univariate analysis, with higher ALT activity and absence of immunosuppressive therapy or past bone marrow transplantation. The ideal candidate for antiviral therapy is an immunocompetent, young, female with low HBV viral load and higher ALT activity. Similar observations were previously reported [4,6,12,22].

We did not confirm the influence of presence drug-resistant HBV variants on NAs treatment efficacy in naïve patients. The Polish National Health Service requirement of a pre-treatment drug resistance test during qualification to antiviral therapy has no justification in our data or in commonly accepted knowledge. We did not find any host- or virus-related causes of HBV drug-resistant variants except for previous insufficient NAs therapy. The main HBV-related predicting response factor was low viral load. Host-related factors of good antiviral response predictors were young age, female sex, high ALT activity, and immunocompetence. Our study did not confirm the role of IL-10 promoter region polymorphism in efficacy of NAs therapy in CHB patients. Natural polymorphism of the host immune system genes seems to be an essential factor affecting elimination of varied pathogens and needs further investigation.
Acknowledgments

Magda Rybicka is the recipient of the European Social Fund in the framework of the project “InnoDoktorant” – Scholarships for PhD students, 5th Edition.

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Conflict of interest

None declared.