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ORIGINAL PAPER

Antiviral drug resistance rates among patients with chronic hepatitis B infection

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ABSTRACT

Introduction and aim. Chronic hepatitis B infection (CHB) affects millions of people around the world. Many clinicians find it challenging to choose therapeutic agents due to the mutations that occur in the hepatitis B virus (HBV) that cause drug resistance. Thus, the aim of this study was to determine the HBV resistance rates against the currently recommended first-line therapies in the region of our country where HBV prevalence is high.

Material and methods. A total of 96 patients (56 men and 40 women) with HBV infection were enrolled in the study. The serum samples collected from those were analyzed with real-time polymerase chain reaction analysis followed by pyrosequencing (PyroStar HBV Drug Resistance Test, Altona Diagnostics, Germany) for drug resistance mutations associated with lamivudine, adefovir, telbivudine, entecavir, and tenofovir.

Results. HBV drug-resistance mutations were investigated in 80 treatment-naïve and 16 treatment-experienced patients (6 entecavir, 4 PEGylated-interferon, 4 tenofovir, 2 lamivudine). None of the HBV-DNA samples had mutations cause to drug resistance were detected in any codons regions that were analyzed.

Conclusion. Antiviral resistance poses serious obstacles for clinicians in the treatment of CHB. Determining whether antiviral resistance exists in HBV is critical to choose the appropriate treatment agent.

Keywords. antiviral agents, antiviral drug resistance, chronic hepatitis B, hepatitis B

Introduction

Hepatitis B virus (HBV) infection is still considered a significant global health problem. Even though the hepatitis vaccine has been used for approximately thirty years, the global prevalence of chronic HBV infection has declined slightly.[1,2] According to the World Health Organization (WHO), approximately 296 million people had chronic HBV infection (CHB) in 2019 and it is estimated that 63 million new cases and 17 million HBV-related deaths will occur between 2015 and 2030.[3] Acute HBV infection leads to CHB in 5%-10% of adults, which may lead to cirrhosis, hepatic decompensation, hepatocellular carcinoma, and thus death.[2,4] The main goal of the treatment in CHB is to reduce the development of those complications. Currently, international guidelines recommend PEGylated-interferon (PEG-IFN) and nucleos(t)ide analogs (NAs) as first-line therapies. NAs include lamivudine (LAM), adefovir (ADV), telbivudine (TBV), entecavir (ETV), and tenofovir (TDF).[5,6] Those treatment options aim to stop the progression of the disease by suppressing the replication of HBV.[5]

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However, CHB requires long-term treatment which leads to mutations thus emerging drug resistance.[2]

Reverse transcriptase (RT) is the major enzyme that is required for viral replication in HBV. However, RT promotes errors during replication generated at a rate of approximately 3×10^{-5} mutations per nucleotide per year which is 10-fold greater than that of other DNA viruses. These mutations change the conformational structure of RT and consequently lead to drug resistance in NAs.[7] Furthermore, those mutations may become severe obstacles for patients with CHB and mostly force clinicians to change the drug, prolonging the treatment duration and thus increasing the costs.

Our country is located in the middle endemic region for HBV with a prevalence of 2-8%, similar to Southern Europe and the Middle East countries.[8] The prevalence is also higher in the eastern region including the city where we conducted this current study.[8]

Aim

Thus, the aim of this study was to determine the resistance rates of the currently recommended first-line therapies against HBV.

Materials and methods

Ethical approval

All of the patients enrolled in this study provided written informed consent. The research was conducted ethically in accordance with the Declaration of Helsinki (2014). This study was approved by the local ethics committee (Van Yuzuncu Yil University Faculty of Medicine Invasive Clinical Trials Ethics Committee - Van. Date: 13.03.2014 decision number: 06).

Study design

A total of 96 patients (56 men and 40 women) were admitted to the gastroenterology clinic in a referral university hospital in Eastern Anatolia for one year period and were diagnosed with CHB (hepatitis B surface antigen (HBsAg) positive by blood test and HBV-DNA positive by polymerase chain reaction (PCR) for more than six months) were enrolled in the study.

Patients' HBV-DNA, HBsAg, hepatitis B "e" antigen (HBeAg), alanine transaminase (ALT), and aspartate transaminase (AST) results were recorded. Those with ALT or AST levels above 40IU/L in the serum samples of the patients were considered elevated. HBeAg and HBsAg levels were detected by the enzyme-linked immunosorbent assay (ELISA) technique (Cobas 401, Roche, Germany), and HBV-DNA was detected by a real-time quantitative PCR kit (QIAsymphony, Qiagen, Germany) with a lower detection limit of 100 IU/mL, ALT and AST levels were detected by Architect c8000 device (Abbott, ABD) in patients' plasma samples according to the manufacturer's instructions.

The Pyrosequencing method of DNA sequencing was used to analyze antiviral resistance in HBV-DNA-positive patients. EZ1 Virus Mini Kit v2 (Qiagen, Hilden, Germany) was used to isolate viral DNA from the serum samples. DNA was extracted according to the manufacturer's instructions using an EZ1 Advanced Instrument (Qiagen, Hilden, Germany). The final elution volume of 50 µL containing viral DNA from each sample was stored at -20°C for long-term usage.

Patients were categorized into three groups according to their HBV-DNA levels; 10^3 to 10^5 IU/ml in group 1, 10^5 to 10^6 IU/ml in group 2, and more than 10^6 IU/ml in group 3. Patients were categorized according to age groups as 16-24 years, 25-49 years and, 50 years and older.

The PyroStar HBV Drug Resistance Test consists of two steps: HBV real-time PCR analysis and pyrosequencing. Two HBV real-time PCR analyses were performed, the first (PCR-1) to detect mutations in codons 169, 173, 180, 181, 184, and 194, and the second (PCR-2) for mutations in codons 202, 204, 236, and 250 of the HBV polymerase gene. The PCR products were then sequenced with pyrosequencing primers to detect HBV drug resistance mutations. The forward primers used in both PCR reactions (PCR-1 and PCR-2) were biotinylated, resulting in a biotinylated amplification product that binds to streptavidin-coated Sepharose beads to isolate single-stranded DNA (ssDNA), and pyrosequencing was carried out with sequence primers. After amplification of ten codon regions containing potential mutations by real-time PCR, ssDNA templates of positive samples hybridized to six different sequence primers were incubated with the enzymes, substrate, and dNTPs. The enzyme mixture includes DNA polymerase, ATP-sulfuryl, luciferase, and apyrase, substrate mixture comprising adenosine 5'-phosphosulphate and luciferin, and each dNTP (dATPαS, dTTP, dCTP ve dGTP) were added to the wells of a Pyromark Q24 cartridge and placed in the Pyromark Q24 (Qiagen) workstation. The DNA sequences of the ten codon regions of each positive sample were analyzed by comparing them with the sequences of the wild-type and the mutant type-with HBV drug-resistance mutation. Mutations that cause antiviral drug resistance, mutant and wild type codons and which antiviral resistance develops as a result are given in Table 1.

The demographic data of the patients and the determined hepatitis B antiviral drug resistance mutations were recorded.

Statistical analysis was performed using Minitab ver.14 (Minitab Statistical Software LLC, Chicago IL USA). The events of interest were reported with mean and standard deviation. To compare proportions between groups, the Chi-square test was used. In all statistical tests, a significance level of 5% ($p<0.05$) was used.

Table 1. Mutations that cause antiviral drug resistance, mutant and wild type codons and which antiviral resistance develops as a result

Mutation	Wild Type Codon	Mutant Codon	Antiviral Resistance
I169T	ATA, ATT	ACA, ACT	Entecavir
V173L	GTG	CTG	Lamivudine
L180M	TTG, CTG	ATG	Lamivudine, Entecavir, Telbivudine
A181V	GCT	GTT	Lamivudine, Adefovir, Tenofovir
T184S	ACT	AGT, TCT	Lamivudine, Entecavir
A194T	GCT	ACT	Telbivudine
S202I	AGC, AGT	ATC, ATT	Entecavir
M204V/I	ATG	GTG, ATC/T/A	Lamivudine, Tenofovir, Telbivudine, Entecavir
N236T	AAC, AAT	ACC, ACT	Adefovir
M250V	ATG	GTG	Entecavir

Results

A total of 96 patients (56 men and 40 women) who were diagnosed with CHB infection were enrolled in the study. The patients' mean age was 37.3±16.7 years (minimum:16; maximum:90). The time elapsed after CHB diagnosis was from one month up to sixteen years (mean: 4 years). HBV drug-resistance mutations were investigated in 80 treatment-naïve and 16 treatment-experienced patients (6 ETV, 4 PEG-IFN, 4 TDF, 2 LAM) (Table 2). No significant difference was found between genders among HBV-DNA levels groups (p=0.175).

Table 2. Antivirals that treatment-experienced patients used and their treatment durations

Patient	Antiviral	Treatment duration
Patient 1	PEGylated-Interferon	2 months
Patient 2	PEGylated-Interferon	3 months
Patient 3	PEGylated-Interferon	5 months
Patient 4	PEGylated-Interferon	4 months
Patient 5	Lamivudine	1 year
Patient 6	Lamivudine	1 year
Patient 7	Entecavir	2 months
Patient 8	Entecavir	5 years
Patient 9	Entecavir	1 year
Patient 10	Entecavir	3 months
Patient 11	Entecavir	6 months
Patient 12	Entecavir	1 year
Patient 13	Tenofovir	3 months
Patient 14	Tenofovir	3 months
Patient 15	Tenofovir	2 years
Patient 16	Tenofovir	1 year

HBeAg was detected positive in 41 (42.8%) patients (19 men and 22 women). HBeAg positivity was found to be more frequent among women (22/40 (55%) vs. 19/56 (34%); p=0.040). Also, it was found that HBeAg positivity was significantly lower in patients older than 50 years compared to younger counterparts (7/24 (29.2%); 34/72 (47%)) and it was highest in patients younger than 25 (19/27; p<0.019). AST levels were high in 49 patients (33 men, 16 women) and ALT levels were high in 47 patients (31 men, 16 women) (Table 3). No significant differences were found between genders and AST or ALT levels.

Table 3. ALT and AST levels and their distribution by gender[a]

	AST levels			ALT levels		
Gender	High n (%)	Low n (%)	p	High n (%)	Low n (%)	p
Men	33 (67%)	23 (49%)	0.067	31 (66%)	25 (51%)	0.65
Women	16 (33%)	24 (51%)		16 (34%)	24 (49%)	
Total	49	47		47	49	

[a] ALT or AST levels above 40 IU/L in the serum samples of the patients were considered elevated; AST – aspartate transaminase; ALT – alanine transaminase

In terms of the relationship between HBV-DNA levels and HBeAg positivity; the difference between the 1st and the 3rd groups and the 2nd and the 3rd groups was statistically significant (p<0.001). And in terms of HBV-DNA levels and elevated levels of liver enzymes, between the 1st and 3rd groups for both ALT and AST, it was found to be a statistically significant difference (p=0.030 and p=0.048 respectively). Patients' HBV-DNA levels and their correlation between HBeAg, AST, and ALT levels are shown in Table 4.

Table 4. Patients HBV-DNA levels and the correlation between HBeAg, AST, and ALT levels [a]

	HBeAg			AST			ALT		
HBV-DNA (IU/ml)	Positive n (%)	Negative n (%)	p	High n (%)	Normal n (%)	p	High n (%)	Normal n (%)	p
10^3–10^5 (n=26)	4 (15.4%)	22 (84.6%)	<0.001	8 (30.8%)	18 (69.2%)	0.049	7 (26.9%)	19 (73.1%)	0.03
10^5–10^6 (n=13)	3 (23.1%)	10 (76.9%)		8 (61.5%)	5 (38.5%)		7 (53.8%)	6 (46.2%)	
>10^6 (n=57)	34 (59.7%)	23 (40.3%)		33 (57.9%)	24 (42.1%)		33 (57.9%)	24 (42.1%)	
Total (n=96)	41 (42.7%)	55 (57.3%)		49 (51%)	47 (49%)		47 (49%)	49 (51%)	

[a] HBeAg – hepatitis B "e" antigen; AST – aspartate transaminase; ALT – alanine transaminase

All the HBV-DNA samples were genotype D and no mutations caused to drug resistance were detected in any codons regions that were analyzed.

Discussion

The ideal target for HBV therapy, defined as a functional cure, is the sustained loss of detectable HBsAg and HBV DNA in serum after a finite course of treatment.[9] However, it may take years to reach that goal, such that only 3% to 11% of the patients treated with PEG-IFN and only 1% to 12% were treated with NAs for 5 to 7 years achieve it.[10] Besides, prolonged treatment time with those agents may increase the likelihood of side effects and thus decreases the treatment success. Nevertheless, the greatest danger of long-term treatment is that it increases the risk of developing resistance to these drugs.

Drug-resistant HBV variants have been compelling for clinicians through the years. As a result, although many antiviral agents have been used for CHB treatment, current international guidelines recommend only TDF and ETV, those associated with high barriers against HBV resistance.[5]

PEG-IFN has limited utilization in CHB treatment due to its side effects (i.e. bone marrow suppression and exacerbation of existing neuropsychiatric symptoms such as depression), and as well as administration way.[11] On the other hand, PEG-IFN has a finite treatment duration and no drug resistance was reported until this time.[10] Four of the patients in the current study received PEG-IFN and no PEG-IFN resistance was detected in any patients, consistent with previous data.

Although current treatment guidelines recommend either PEG-IFN or NAs, because of the narrow usage area of PEG-IFN, NAs are the preferred treatment option in most patients.[11] NAs do not affect covalently on closed circular DNA of HBV. Therefore, the treatment requires long-term administration which could cause drug-resistant mutations in the viral enzyme RT.[12] This is especially evident in LAM, which is safe, well-tolerated, and the first NA approved for the treatment of HBV infection. However, LAM resistance rates were found to be extremely high around the world. Since the LAM resistance rate could be 23% after one year of treatment and could reach up to 70–80% after 4–5 years.[13,14] In a multicenter study conducted on 1568 patients in 18 European countries, the LAM resistance rate was found to be 60.1%.[15] Another study conducted in China was performed on 1223 HBV-infected patients and the drug resistance rate was found to be 46.5% for LAM.[16] In our country, LAM resistance was also found to be high. In a recent study performed by Alacam et al., drug resistance mutations were found 46.86% in patients with HBV infection and the resistance rate for LAM was found 36.79%.[17] Due to those high resistance rates, LAM use has decreased over the years.[13,14] Nevertheless, none of our patients had mutations related to LAM resistance.

ADV is one of the other options that can be used for CHB infection. ADV resistance is lower than the LAM resistance. A previous study conducted by Hadziyannis et al. found ADV resistance rate of 5.9%, with more than 144 weeks of ADV monotherapy.[18] Another comprehensive study conducted in China found that 11.4% of the patients had ADV-resistant HBV variants.[16] ADV is both used against treatment-naïve and LAM-resistant HBV variants.[19,20] Unfortunately, previous studies have shown that LAM resistance could facilitate ADV resistance. A recent study conducted in LAM-resistant and treatment-naïve CHB patients found that after two years of ADV treatment ADV resistance emerged considerably higher compared to treatment-naïve CHB patients.[21] LAM, ADV, and TBV are considered agents with low genetic barriers due to the number of mutations required to confer resistance are low. A recent study conducted a 1-year trial of TBV found that 4.5% of patients who received TBV treatment for 48 weeks developed resistance.[22] Resistance rates against TBV are lower compared to LAM and ADV, however, after 2 years of treatment, these rates reach 17 percent.[6] Therefore, it is recommended that patients resistant to TBV should be immediately switched to TDF monotherapy.[6,23] Although many CHB patients are still being treated with other NAs, current guidelines recommend using NAs with a high barrier to resistance such as ETV or TDF because of their high efficacy in virological suppression and the lowest risk of the emergence of resistance.[5,6] Moreover, to date, there has been no confirmed resistance to TDF was detected, even after long treatment durations. Suzuki et al. followed up with 40 patients that received TDF monotherapy or combination for a median of 45 months and found no resistance against it.[12] Snow-Lampart et al. also reached the same conclusion in their study conducted with 641 patients after receiving 144 weeks of TDF monotherapy.[24] In contrast to TDF, development of resistance to ETV has been reported, but this resistance is significantly lower compared to other NAs resistances.[25] None of our patients had resistance to ETV. In support of this, a recent study found only 1.2% of the treatment-naïve patients enrolled to their study had ETV resistance.[26] However, similar to ADV, the rate of resistance to ETV is higher in LAM-resistant patients than in patients who have not used drugs before.[27,28] Zoulim et al. found that although ETV resistance rate is very low in treatment-naïve patients, that rate increases to 51% of the patients.[28]

This study has also several limitations to be considered. Although, it was conducted in the largest referral hospital in the region with the highest prevalence of HBV infection in our country, it was a single-center study, so our findings cannot be generalized. Another potential limitation relates to our investigation was our relative small sample size. Particularly in light of increasing resistance rates among HBV being a worldwide concern, we believe that this issue clearly requires further investigation, and new and comprehensive studies in this field are increasingly important.

Conclusion

In this study, we assessed the genetic variability of HBV in Eastern Anatolia and HBV genotype D was the determined genotype in all patients and this finding is in line with the general data of our country.[29] CHB treatment has been posing an obstacle for clinicians and patients due to increasing antiviral resistance rates. Our findings on drug resistance mutations showed that either treatment-naïve or treatment-experienced CHB patients have no HBV polymerase resistance mutation rate which means resistance while initiating treatment was

not seem to be a major problem in our region. However, further monitoring of the newly diagnosed HBV-infected patients should be continued, in order to evaluate the presence of transmitted drug resistance and its influence on the response to treatment.

Declarations

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Author contributions

Conceptualization, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Methodology, S.Ö., Y.B., A.Ö., M.P.; Software, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Validation, S.Ö., Y.B., A.Ö., M.P.; Formal Analysis, S.Ö., Y.B., A.Ö. and M.P.; Investigation, S.Ö., Y.B., A.Ö. and M.P.; Resources, S.Ö., Y.B., A.Ö. and M.P.; Data Curation, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Writing – Original Draft Preparation, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Writing – Review & Editing, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Visualization, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Supervision, S.Ö., Y.B., A.Ö., M.P., A.Ö. and C.A.; Project Administration, S.Ö., Y.B., A.Ö. and M.P.

Conflicts of interest

No conflict of interest was declared by the authors.

Ethics approval

This study was approved by the local ethics committee (Van Yuzuncu Yil University Faculty of Medicine Invasive Clinical Trials Ethics Committee – Van. Date: 13.03.2014 decision number: 06).

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