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Hepatitis B Virus Carrying Drug-resistance Compensatory Mutations in Chronically Infected Treatment-naive Patients

Tedavi Almamış Kronik Hepatit B Olgularında İlaç Direnci İlişkili Kompansatuvar Mutasyonlar

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ABSTRACT

Objective: The prevalence of hepatitis B virus (HBV) is highly variable throughout the world. Geographical regions are classified according to the prevalence of hepatitis B surface antigen in the general population as high (>8%), moderate (2-7%), and low endemicity (<2%). Turkey has a moderate endemicity level of HBV infection which is a serious health problem. Currently, there are various nucleos(t)ide analogues with anti-HBV activity and they are mostly used in the treatment of chronic hepatitis B (CHB) and cirrhosis. The risk of drug resistance increases because these drugs are still being used as monotherapy. It has been reported that HBV drug resistance-related mutations can occur also in patients who are classified as treatment-naive and who have not received any oral anti-HBV treatment.

Materials and Methods: This prospective and descriptive epidemiological study aimed to determine the genotype/subgenotypes of HBV and to investigate the drug resistance mutations in treatment-naive CHB patients. The study included 149 CHB patients who had no chronic co-infections, and have not received treatment for CHB infection. In 53 of the samples collected from the patients, the amount of viral DNA was enough for sequence analysis to search for drug resistance. BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) was used for sequencing of the serum samples from these patients and drug resistance mutations were determined and genotype/subgenotype detection was performed.

Results: The mean viral load value was calculated as $9.84x10^6$, and there was no primary drug resistance in any of these 53 samples which were sequenced. There were compensatory resistance-related amino acid changes in 19 samples. Genotype D was determined as HBV in all cases.

Conclusion: The early detection of drug resistance-related mutations can be important in determination of treatment protocol, and prevention of unnecessary drug use, complications, and economic loses.

Keywords: Hepatitis B virus, naive patients, drug resistance, compensatory mutation

ÖZ

Amaç: Dünya çapında hepatit B virüs (HBV) prevalansı oldukça değişkendir. Yüksek (>%8); orta (%2-7) ve düşük (<%2) endemik bölgeler içinde sınıflandırılmıştır. Türkiye HBV enfeksiyonu açısından orta endemisiteye sahip olup ülkemiz için ciddi bir halk sağlığı sorunudur. Günümüzde anti-HBV aktivitesi olan çeşitli nükleozit analogları mevcuttur ve kronik hepatit B (KHB) ve siroz tedavisi için çoğunlukla kullanılmaktadır. Bu ilaçlar monoterapi şekilde kullanıldığından ilaç direnci riski artmaktadır. HBV ilaç direnci ile ilişkili mutasyonların, herhangi bir oral anti-HBV tedavisi almamış naif hastalarda da meydana geldiği bildirilmiştir.

Gereç ve Yöntemler: Prospektif, tanımlayıcı temelinde gerçekleştirilen bu epidemiyolojik çalışmada, naif KHB hastalarında ilaç direnç mutasyonlarının araştırılması ve HBV genotip/subgenotiplerinin belirlenmesi amaçlanmıştır. Çalışmaya KHB olduğu bilinen, başka bir kronik koenfeksiyonu bulunmayan, KHB enfeksiyonu için tedavi almamış 149 hasta dahil edilmiştir. Hastalara ait örneklerden 53'ünde viral DNA miktarı ilaç direnci araştırmasında sekans analizi için yeterli olmuştur. Hastalara ait serum örneklerinde BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., ABD) kullanılarak yapılan sekanslama yöntemi ile ilaç direnç mutasyonları araştırılmış, genotip/subgenotip tayini yapılmıştır.

Bulgular: Katılımcıların ortalama viral yük değeri $9,84x10^6$ olarak hesaplanmış, sekanslaması yapılan 53 örneğin hiçbirinde primer ilaç direnci saptanmazken, 19 olguda kompansatuvar direnç ile ilişkili aminoasit değişiklikleri tespit edilmiştir. Olguların tamamında genotip D olarak belirlenmiştir.

Sonuç: KHB tedavisindeki amaç hastalığın progresyonunu önlemek ve sağ kalımı sürdürmektir. İlaç direnci ile ilişkili mutasyonların erken dönemde tespiti, tedavi protokolünün belirlenmesinde yol gösterici olması, gereksiz ilaç kullanımı, komplikasyon gelişimi ve ekonomik kayıpların önlenmesi açısından önemli olabilir.

Anahtar Kelimeler: Hepatit B virüs, naif olgular, ilaç direnci, kompansatuvar mutasyon

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Introduction

Hepatitis B virus (HBV) is a viral factor leading to acute and chronic infections by affecting the liver (1). Even though an effective hepatitis vaccine has been commonly used, approximately 2 million individuals (almost one third of the world's population) encounter the hepatitis virus and it is known that HBV is a growing global public health problem (2). Probably 240 million people have chronic HBV infection and more than 686.000 individuals die every year due to hepatitis B infection-related diseases such as cirrhosis and liver cancer (1). It has been specified in studies conducted in our country that the prevalence of hepatitis B surface antigen varies between 0.8% and 14.3% and it has been reported that our country has a moderate endemicity level among other regions of the world (3).

HBV can lead to different clinical outcomes such as asymptomatic infections, fulminant hepatitis, inactive carrier state, and even life-threatening diseases such as liver cirrhosis and liver cancer. Therefore, early detection and treatment of HBV is crucial. Non-specific factors, such as age, and specific genetic factors in the host as well as genetic features of the virus can affect the prognosis of the HBV infections (4). Genetic variance can occur due to the genotypic differences in carriers or mutations in the infected host (5,6). Although it has not yet been clarified, findings support that genotypic differences have effects on the HBV pathogenicity and the clinical course of the infection. Therefore, determination of the genotype in the beginning of the disease can contribute to clinical approaches in a more conscious way (4). It has been determined that there are 10 different HBV genotypes (from A to J) in addition to the recently defined genotypes (5,6). However, there have been a limited number of studies on HBV genotype distribution in Turkey. In the work done in the obtained data, genotype D and subgenotype D1 have been found to be a widely prevalent genotype in Turkey (7,8,9).

Developments in viral techniques used in the diagnosis of chronic HBV infections ensure that individual treatments can be more accurately decided. Recently, hepatitis treatment is better managed by the application of molecular techniques, which can quantitatively detect HBV DNA, genotype detection, and determination of the antiviral resistance. It is very important that the right patient is treated conveniently and the right drug is used for the sake of the future of both the patient and the drug. Therefore, the treatment goals and treatments should be accurately determined (10,11).

Recently, there have been various approved chronic hepatitis B (CHB) treatments which prominently decrease the mortality and morbidity rates. They include two interferons (IFNs) (conventional and pegylated alfa-2a) analogues and five nucleoside/nucleotide analogues (NAs): lamivudine (LAM), telbivudine (TBV), entecavir, adefovir (ADV), and tenofovir. NA inhibits the activity of HBV polymerase and suppresses its replication by binding it competitively. However, the drug resistance is a common problem in long-term treatments (12). Furthermore, recent studies showed that there is an antiviral resistance even in HBV isolated from non-treated patients (13).

In this study, the pol and s gene kinetics İN treatment-naive HBV patients were evaluated by using the sequencing technique and drug resistance-related mutations were examined. The aim of the study was to determine the HBV genotype and subgenotypes and thus contribute to the epidemiological data.

Materials and Methods

In this prospective and descriptive epidemiological study, we included a total of 149 patients who had no any infection except CHB and who were treated for CHB between January 2012 and May 2013. Blood samples obtained from the patients were used for routine laboratory tests and then remaining blood serum samples were stored at -80 ºC till they were used. The amounts of viral DNA in 53 samples were sufficient for drug resistance sequence analysis assay. The demographic and clinical data of participants were obtained from the hospital records. The ethical approval of this study (15/10/2015-12797) was obtained from Sakarya University.

HBV DNA levels were measured by the real-time polymerase chain reaction (RT-PCR) technique with the help of a Cobas TaqMan 48 kit (Roche Diagnostics, USA). The degenerate primers were used to amplify the 16S gene, from HBV samples and from isolates. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and used for direct sequencing using a BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA), and an ABI-3100 at the Center for Comparative Genomics at the University of Iowa (Iowa City, Iowa, USA). The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank. Alignments were carried using CLUSTALW [http://clustalw.ddbj.nig.ac.jp/top-e.html] and neighbor-joining tree was formed. Statistical differences were analyzed by DNA polymorphism levels within locations and mutations were summarized in haplotype diversity (h) and nucleotide diversity (π) with standard deviation. Population expansions were assessed by neutrality tests implemented in DnaSP v.5.10.01, for the Tajima's D and the Fu and Li's F.

Results

Gene sequences of PCR products in 53 patients (41 male and 12 female) were obtained by using PCR and sequencing techniques. The amount of DNA samples in the rest of patients was not sufficient for sequence analysis. The average age of the patients was 49.3 years (range: 24-74). The average viral load was $9.84x10^6$ IU/mL (range: $1.346x10^1$ IU/mL-$4.383x10^8$ IU/mL. Some demographical characteristics and laboratory findings of patients are shown in Table 1. All the 53 patients had genotype D. Subgenotype distribution was as follows: 46 patients had genotype D1, 4 patients had genotype D2, and 3 patients had genotype D3. There was no primary drug resistance in any of the patients (Table 1). There were amino acid alterations in 19 patients and these alterations were associated with compensatory resistance which had roles in viral replication repair and increased viral loads (Table 2). It was detected that there were compensatory changes associated with only TBV, LAM and ADV, and only ADV in 4, 13, and 2 patients, respectively.

Discussion

The final goal of CHB treatment is to prevent the progression of the disease and to maintain survival. It is possible to achieve these goals by suppressing HBV replication and maintaining this

suppression (14). Recently, seven antiviral agents have been approved in order to be used in the CHB infection treatment. Two of them were IFN-α(IFN/pegylated IFN) analogues and five of them were NAs. NAs suppress HBV replication by affecting the reverse transcriptase enzyme. The most important issue during long-term CHB treatment with NAs is the mutagenesis which is responsible for the antiviral agent resistance. Amino acid alterations in the HBV reverse transcriptase cause NA resistance which is an important problem in CHB treatment (6,14). Even though it is specified that there is no need to examine drug resistance in patients who have not been previously treated (10,14,15), there can be spontaneous drug resistance mutations in treatment-naive patients (6). NA resistance mutations can occur in non-treated patients because of the viral and patient factors. The reasons for the variance of resistance in non-treated patients are: 1) these mutations can naturally occur during the replication (10^{-5}-10^{-4} substitution/base/cycle), 2) the patient can be infected by a mutant virus from another patient who was previously treated, and 3) the patient can be directly and unconsciously exposed to equivalent components with anti-HBV activity (12,16). Thus, resistance mutations can be observed in non-treated patients. These mutations can be primary drug resistance mutations (drug unresponsiveness) or they can increase the replication capacities of reduced resistant HBV variances (11).

In recent studies conducted with non-treated patients in different countries, it was shown that the frequencies of HBV strains which had clinically important drug resistance mutation (NA resistance) were between 0.5% and 1% (17,18,19,20,21,22,23,24,25). In our country, Akarsu et al. (26) evaluated 71 inactive HBV carriers who have not received any treatment and they detected YMDD variants (18.3%) in 13 cases. Yıldız et al. (27) detected a primary resistance against LAM in 8 of 202 LAM-naive CHB patients (4%). Sayan et al. (28) evaluated 88 treatment-naive CHB patients and they showed that there were amino acid alterations in HBV polymerase genes in 17 of these patients (19%). Ergünay et al. (29) evaluated 30 CHB patients who were not previously treated and they determined HBV NA resistance-related mutations in 3 of them (10%).

Table 1. Demographical characteristics and laboratory information of 53 patients whose DNA was sequenced

Clinical factors	Value
Male/Female	41/12
Average age (year)	49.3 years
HBeAg positive (n)	26
Average ALT (U/mL)	16.7
Average AST (U/mL)	39.8
Average HBV DNA (IU/mL)	$9.84x10^6$
HBV genotype (subgenotype)	46 (D1) 4 (D2) 3 (D3)

HBeAg: Hepatitis B envelope antigen, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HBV: Hepatitis B virus

Table 2. Number of compensatory mutation cases in treatment-naive hepatitis B virus patients

Reverse transcriptase mutations (compensatory)	Antiviral agent	Case number
Q149K	LAM and ADV	4
Q215S	LAM and ADV	4
Q215H	LAM and ADV	4
L91I	TBV	4
Q249K	LAM and ADV	1
N238D	ADV	1
V214A	LAM and ADV	1

LAM: Lamivudine, ADV: Adefovir, TBV: Telbivudine

In our study, there was no primary drug resistance in any of the 53 patients. In order to determine the nucleotide alterations in targeted region of the viral genome, DNA sequence analysis method was applied by using PCR products. This technique has an advantage of showing all mutations in the amplified region and it is accepted as the gold standard for the detection of nucleotide alterations. However, mutant genomes should constitute at least 20% of the whole viral population in order to detect all of the mutations in the genome directly by using sequence analysis. In our study, it is possible that we did not detect primary drug resistance amino acid alterations because of their low levels. Recent and new generation sequencing systems can be used to analyze the whole viral genomes and thus all viral genome pool of infected people can be examined. However, these approaches cannot be easily used in all institutions because they require equipments and experienced staff (29,30). Furthermore, emergence of resistance variants against approved NAs is the main reason for treatment failure in CHB infections. Therefore, the treatment can be regulated timely with the help of the early detection of these mutations and thus aggravation of the disease can be prevented. Meanwhile, early detection of these mutations can also contribute to detection of compensatory mutations which can repair the viral replication and increase the resistance risk against other drugs (16).

Population sequencing method can be used to detect the known NA resistance mutations as well as compensatory mutations. It has been reported that rtQ215H/Q/P/S compensatory mutations are frequently observed in CHB patients who receive both NA-naive and LAM and/or ADV treatments (11,12). In our study, there were compensatory mutations in 19 patients and 4 of them had rtQ149K compensatory mutation which was associated with LAM and ADV treatment. Other compensatory mutations were Q215S mutation associated with LAM and ADV (4 patients), Q215H mutation associated with LAM and ADV (4 patients), rtL91I mutation associated only with TBV (4 patients), Q249K mutation associated with LAM and ADV (1 patients), N238D mutation associated with only ADV (1 patients), and V214A mutation which was associated with LAM and ADV (1 patient) (Table 2).

Compensatory mutations which can repair the viral replication and increase the viral load and particularly the mutations related to drug resistance in NA target region of the HBV polymerase gene can be associated with treatment failure. Compensatory mutations can lead to unnecessary or incorrect medication changes in CHB patients. Mutation detection in HBV patients can have important effects on the development of different treatment strategies (31).

It has been specified that there are at least 10 HBV genotypes (A-J) in case of differences more than 8% in the HBV genome. Some genotypes are divided into subgenotypes in case there is

a 4-8% difference between nucleotide sequences. Recent data has shown that genotypes are determining factors affecting the severity of the liver disease and the response against the antiviral drug (7). Furthermore, HBV genotype D is the most commonly determined genotype in our country and the most commonly observed subgenotype is the genotype D1. Even though Kaklikkaya et al. (7) have reported that the most commonly observed genotype was genotype D2, other studies claimed that the genotype D1 was the most frequently detected one in our country. In our study, all patients were classified as HBV genotype D and the most frequently detected genotype was genotype D1. Genotype D2 and genotype D3 were the other genotypes that can be observed in Turkey.

NA combinations are important in treatment-resistant patients and initial treatment selection and subsequent treatment decisions should depend on the resistance rates (6). Consequently, early detection of antiviral drug resistance-related mutations can be important in determination of the most convenient treatment protocol. Thus, toxicity and economic losses due to unnecessary drug use can be prevented and severe complications can be reduced (32,33). Although we did not detect such mutations in our study, there are other studies in which drug resistance mutations are detected in treatment-naive CHB patients. Furthermore, it will be clinically useful to determine the resistance profile since treatment-naive CHB patients develop primary drug resistance against LAM and ADV (29). Further studies investigating mutations in the viral polymerase region and determining the clinical importance of these mutations in treatment-naive patients are warranted.

Ethics

Ethics Committee Approval: The ethical approval of this study (15/10/2015-12797) was obtained from Sakarya University.

Peer-review: External and Internal peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Mustafa Altındiş, Concept: Mustafa Altındiş, Design: Mustafa Altındiş, Ferhat Gürkan Aslan, Mehmet Köroğlu, Data Collection or Processing: Mustafa Altındiş, Ferhat Gürkan Aslan, Mehmet Köroğlu, Leyla Demir, Mustafa İhsan Uslan, Savaş Aslan, Mehmet Özdemir, Analysis or Interpretation: Mustafa Altındiş, Ferhat Gürkan Aslan, Mehmet Köroğlu, Ayla Eren, Mahmut Baykan, Literature Search: Mustafa Altındiş, Ferhat Gürkan Aslan, Ayla Eren, Writing: Mustafa Altındiş, Ferhat Gürkan Aslan, Ayla Eren.

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