RESEARCH ARTICLE / ARAŞTIRMA

DOI: 10.4274/mjima.2018.33
Mediterr J Infect Microb Antimicrob 2018;7:33
Erişim: http://dx.doi.org/10.4274/mjima.2018.33

Detection of Hepatitis B Virus Drug Resistance Mutations by Pyrosequencing Method

Hepatit B Virüs İlaç Direnci Mutasyonlarının Pyrosequencing Metodu ile Tespiti

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Abstract

Introduction: The development of drug resistance mutations to nucleos(t)ide analogues during long-term therapy for chronic hepatitis B virus (HBV) infection is a major problem that may lead to treatment failure. The aim of this study was to investigate the HBV drug resistance gene mutations in patients with chronic HBV infection by pyrosequencing method.
Materials and Methods: Between December 2013 and May 2014, serum samples collected from 137 patients with chronic HBV infection, (89 treatment-naive and 48 treatment-experienced), 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 (LAM), adefovir, telbivudine (TEL), entecavir (ETV), and tenofovir (TDF).
Results: Of the 89 treatment-naive patients, one (1.1%) had the rtA194T mutation, associated with reduced susceptibility to TDF. Of the 48 treatment-experienced patients, one (2.1%) had the rtM204I mutation, associated with drug resistance to LAM, TEL, and cross-resistance to ETV. Compensatory mutation rtL180M was observed in two patients (4.2%). The presence of rtM204V combined with rtT184S mutation indicating ETV resistance was detected in one patient (2.1%).
Conclusion: The incidence of drug resistance mutations was 8.3% in treatment-experienced and 1.1% in treatment-naive patients. The use of pyrosequencing technology before and during treatment of patients with chronic HBV infection would contribute to the rapid detection of drug resistance mutations.
Keywords: Lamivudine, entecavir, tenofovir, drug resistance mutations, pyrosequencing method

Öz

Giriş: Kronik hepatit B virüs (HBV) enfeksiyonun uzun süreli tedavisi sırasında nükleoz(t)id analoglarına karşı ilaç direnci mutasyonlarının gelişmesi tedavi başarısızlığına yol açabilen önemli bir sorundur. Bu çalışmanın amacı kronik hepatit B enfeksiyonu olan hastalarda HBV ilaç direnci gen mutasyonlarının pyrosequencing metodu ile araştırılmasıdır.
Gereç ve Yöntem: Kasım 2013 ve Mayıs 2014 tarihleri arasında, kronik hepatit B enfeksiyonu olan 89'u tedavi almayan (naif) ve 48'i tedavi alan toplam 137 hastaya ait serum örnekleri lamivudin (LAM), adefovir, telbivudin (TEL), entekavir (ETV) ve tenofovir (TDF) ile ilişkili ilaç direnç mutasyonlarının tespiti için real-time polimeraz zincir reaksiyonu testi sonrası pyrosequencing metodu (PyroStar HBV Drug Resistance Test, Altona Diagnostics, Germany) ile analiz edilmiştir.
Bulgular: Tedavi almayan 89 hastada, TDF'ye duyarlılığı azaltan rtA194T mutasyonu bir (%1,1) olguda bulunmuştur. Tedavi edilen 48 hastada, LAM ve TEL'e ilaç direnci ve ETV'ye karşı da çapraz dirence sebep olan rtM204I mutasyonu bir (%2,1) olguda tespit edilmiştir. Kompansatuvar mutasyon olan rtL180M iki (%4,2) hastada gözlenmiştir. ETV direncini gösteren rtT184S mutasyonu ile birlikte rtM204V'nin varlığı bir (%2,1) hastada tespit edilmiştir.

Cite this article as: Çimentepe M, Yıldırım D, Kömür S, Şeflek B, Özdoğru Sağdıç D, Yarkın F. Detection of Hepatitis B Virus Drug Resistance Mutations by Pyrosequencing Method. Mediterr J Infect Microb Antimicrob. 2018;7:33.

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Received/Geliş Tarihi: 27.06.2018 **Accepted/Kabul Tarihi:** 20.10.2018

Presented in: This study was presented as a poster at the 1st National Virology Meeting and Workshop held on February 26-28, 2016 in Ankara, Turkey.

Published: 23 October 2018

Sonuç: İlaç direnci mutasyonlarının insidansı tedavi alan hasta grubunda %8,3 ve tedavi almayan grupta %1,1 olarak bulunmuştur. Kronik hepatit B enfeksiyonu olan hastalarda tedavi öncesi ve sırasında pyrosequencing teknolojisinin kullanımı ilaç direnci mutasyonlarının hızlı bir şekilde belirlenmesine katkıda bulunacaktır.
Anahtar Kelimeler: Lamivudin, entekavir, tenofovir, ilaç direnci mutasyonları, pyrosequencing metodu

Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide. Nearly two billion people have been infected with HBV globally and 350 million of them are HBV carrier[1]. Acute HBV infection may progress to chronic hepatitis, which leads to cirrhosis and hepatocellular carcinoma (HCC). Each year, approximately one million people die from cirrhosis and HCC due to chronic HBV[2,3].

Interferons (IFN) and five nucleos(t)ide analogues (NAs) are used in the treatment of chronic hepatitis B. The NAs are lamivudine (LAM), telbivudine (TEL), entecavir (ETV), adefovir (ADV), and tenofovir (TDF). These antiviral drugs are effective in suppressing viral HBV replication and have few side effects[4]. However, long-term antiviral therapy cannot completely eradicate HBV. The clinical benefit of treatment is to maintain suppression of HBV DNA replication by 4-6 log IU/ml Unfortunately, the major limitation of long-term treatment with NAs is the emergence of drug-resistant mutant virus, which is a major factor in treatment failure. Antiviral drug resistance generally results in the progression of liver disease. In addition, drug-resistant HBV mutants may be transmitted to other people. Therefore, the identification of HBV resistance mutations against NAs are important for the selection of appropriate treatment[5,6].

Pyrosequencing is a novel DNA sequencing technology developed at the Royal Institute of Technology and is the first alternative to the conventional Sanger method for de novo DNA sequencing. This method is based on the sequencing-by-synthesis principle and on the detection of pyrophosphate (PPi) released during DNA synthesis. It employs a series of four enzymes to accurately detect nucleic acid sequences during synthesis. Pyrosequencing has opened up new possibilities for performing sequence-based DNA analysis and is especially suitable for single nucleotide polymorphism analysis and sequencing of short stretches of DNA. Pyrosequencing has been successful in both confirmatory sequencing and de novo sequencing[7].

The aim of this study was to investigate HBV drug resistance gene mutations against LAM, TEL, ETV, ADV, and TDF in patients with chronic HBV infection by pyrosequencing method.

Materials and Methods

Between November 2013 and May 2014, a total of 137 patients with chronic HBV infection who were admitted to the Department of Infectious Diseases and Clinical Microbiology, of the Çukurova University Faculty of Medicine were included in the study. Serum samples were obtained from all patients and HBV drug resistance mutations were detected using real-time PCR and pyrosequencing (PyroStar HBV Drug Resistance Testing, Diagnostics Altona, Germany).

Ethics Committee Approval was received from the Ethics Committee of Çukurova University Faculty of Medicine at the beginning of the study (approval no: 11.10.2013 25/26). The demographic data of the patients and the predetermined hepatitis B antiviral drug resistance mutations were recorded.

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)[8].

Pyrosequencing is a DNA sequencing method based on real-time detection of PPi released during DNA synthesis by luciferase assay. 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[8].

The forward primers used in both PCR reactions (PCR-1 and PCR-2) were biotinylated, resulting in a biotinylated amplification product which 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 including 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 with the sequences of the wild-type and the mutant type with HBV drug-resistance mutation.

Results

HBV drug-resistance mutations were investigated by pyrosequencing in 89 treatment-naive and 48 treatment-experienced patients. These 48 cases were previously or currently treated with NAs (11 LAM, 11 TDF, 8 ETV, and the others with various combinations of LAM, TDF, ETV, and IFN). The patients were between the ages of 18 and 77 years old; 52 were female and 85 were male.

The incidence of HBV drug resistance mutations was 1.1% (1/89) in the treatment-naive group and 8.3% (4/48) in the treatment-experienced group. Drug resistance mutations were detected at positions rtL180M (n=2), rtT184S (n=1), rtA194T (n=1), rtM204I (n=1), and rtM204V (n=1).

Among the treatment-naive patients, the rtA194T mutation associated with reduced TDF susceptibility was detected in a 52-year-old male patient.

In the treatment-experienced group, an 18-year-old female patient with pre-existing LAM resistance was treated with LAM (six years), ETV (two years), and IFN (one year). The rtM204I mutation responsible for resistance to LAM and TEL and cross-resistance to ETV was detected in this patient (1/48; 2.1%).

Compensatory mutation rtL180M was detected in two patients (4.2%). One of these patients had been treated with LAM (five years), ADV (one year), and IFN (six months) and already had resistance to LAM and ADV. The other patient received LAM (five years), ADV (1.5 years), TDF (four months), and IFN (two years) and previously showed only LAM resistance.

In the treatment-experienced group, rtM204V combined with rtT184S mutation indicating ETV resistance was detected in one patient (2.1%) that had received LAM-based therapy (five years) (Table 1).

According to the results of Fisher's Exact test, there was a borderline difference in mutation rate between the naive and treated patients groups (p=0.0509).

Discussion

The main goal of antiviral therapy in chronic HBV infection is to clear the virus from the host and prevent progression of liver disease to cirrhosis and HCC. However, HBV may not be completely eradicated due to the presence of cccDNA in the nuclei of infected hepatocytes. Nucleotide analogues like LAM, TEL, ETV, ADV, and TDF target the reverse transcriptase domain of HBV polymerase. NAs exert their antiviral effects by targeting short negative DNA strand synthesis, reverse transcription from pgRNA to negative DNA strand, or DNA-dependent DNA synthesis. NAs are similar in structure to natural nucleotides except for the sugar ring or base group. Therefore, NAs compete with natural nucleotides to bind to viral DNA polymerase and inhibit its activity[9,10].

Table 1. Demographic, laboratory, and clinical data of patients with drug-resistant hepatitis B virus

	Treatment-naive (n=1)	Treatment-experienced (n=4)			
M/F	M	M	F	F	F
Age, years	52	18	18	19	40
HBsAg (IU/ml)	8004	996.8	285.2	387.5	5654
HBeAg	-	+	+	+	-
Anti-HBe	+	-	-	-	+
HBV DNA (IU/ml)	590	1,930,000	170,000,000	1,340,000	715,000
ALT (IU/l)	20	34	104	48	172
AST (IU/l)	19	23	56	41	112
Treatment (duration)	Never treated	LAM (6 years) ETV (2 years) IFN (1 years)	LAM (5 years) ADV (1 years) IFN (6 months)	LAM (5 years) ADV (1.5 years) TDF (4 years) IFN (2 years)	LAM (5 years)
Previously identified drug resistance	-	LAM	LAM+ADV	LAM	-
Detected resistance mutation	rtA194T	rtM204I	rtL180M	rtL180M	rtM204V + rtT184S
Drugs associated with mutations	TDF	LAM, TEL, ETV	LAM, TEL, ETV	LAM, TEL, ETV	ETV, LAM, TEL

M: Male, F: Female, LAM: Lamivudine, TEL: Telbivudine, ETV: Entecavir, TDF: Tenofovir, IFN: Interferon, HBsAg: Hepatitis B surface antigen, HBV: Hepatitis B virus, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

In the present study, HBV drug resistance mutations to LAM, TEL, ETV, ADV, and TDF were detected by pyrosequencing in the serum samples of 137 (89 treatment-naive and 48 treatment-experienced) patients with chronic HBV infection. The rtA194T mutation associated with partial TDF drug resistance was detected in one treatment-naive patient (1.1%). The rtA194T polymerase mutation has been found in HBV/HIV coinfected patients during TDF treatment, suggesting an association with TDF resistance. *In vitro*, this rtA194T polymerase mutation is associated with partial drug resistance against TDF. On the other hand, the rtA194T mutation reduces susceptibility to TDF and impairs viral replication capacity of HBV constructs *in vitro*, possibly explaining the low occurrence of this mutant form in clinical practice[11-13].

The rate of rtA194T mutation in our study was 1.1%, similar to the rate of 0.7% reported by Salpini et al.[14] in Rome. Pastor et al.[15] detected rtA194T in 1 of 14 treatment-naive patients with chronic HBV infection in Strasbourg, France. In China, Zhao et al.[16] detected spontaneous HBV resistance mutations other than rtA194T (such as rtM204V/I, rtL180M, rtT184G, rtS202I) in 8.9% of their treatment-naive group. In studies conducted in Turkey, Sayan et al.[17] detected rtA194T mutation in 2.2% of 88 treatment-naive patients with chronic hepatitis B, while Ergünay et al.[18] reported rtA194T mutation in 3.3% of 42 treatment-naive patients with chronic HBV infection. However, Jiang et al.[19] reported rtA194T mutation in only two of 317 treatment-experienced patients (0.6%). In a treatment-naive patient, rtA194T mutation may occur during chronic infection. The probability of this mutation is usually proportional to the intensity of selection and and the diversity of HBV quasispecies. This shows that there is a natural polymorphism in the chronic hepatitis B population that can predispose to resistance to certain antiviral agents. In addition, the patient may be infected with strains from other patients treated with nucleotide analogues or with TDF resistance[15].

In the presented study, the incidence of drug-resistance mutation in the treatment-experienced group was 8.3% (4/48). One (2.1%) of these patients had rtM204I mutation, which confers LAM and TEL resistance and cross-resistance to ETV. HBV strains carrying rtM204V/I and rtL180M associated with LAM and TEL resistance were also reported to show cross resistance to ETV[20]. The molecular structures of L nucleosides such as LAM and TEL are similar. Therefore, their antiviral resistance mutation profiles are identical. The best known mutation for the L-nucleoside pathway is the amino acid change of methionine (rtM204) to either valine or isoleucine (V/I) at position 204 in the YMDD catalytic motif of the reverse transcriptase. Alone, these major mutations may confer resistance. However, they are often found with compensatory mutations such as rtL180M/C and rtV173L. The resistance mutations associated with LAM are primarily rtM204V/I/S, rtA181T, rtL180M/C, rtV173L, rtL80V/I, rtI169T, rtT184S/G, and rtQ215S[9,21,22].

The rtM204V/I mutation has been found to reduce *in vitro* sensitivity to LAM by 100 fold. While rtM204I substitution may be detected alone, rtM204V/S may be seen together with other mutations[4]. He et al.[23] determined the frequency of rtM204I to be 71.88% and Wen et al.[24] as 29.2%, whereas in Turkey the rates of isolated rtM204I were reported as 12% by Kırdar et al.[25], 9% by Timur et al.[8], 5.8% by Aydoğan et al.[26], 6.4% by Bozdayi et al.[27], and 1.5% by Saran et al.[28].

The compensatory mutation rtL180M is commonly detected with rtM204V/I/S and enhances viral replication, thereby increasing resistance to LAM. However, the presence of rtL180M alone is associated with very low resistance and this secondary mutation usually develops subsequently in HBV strains with the rtM204V/I mutation[13,29,30]. In our study, rtL180M alone was detected in 4.2% (n=2) of 48 treatment-experienced patients. However, rtL180M as a single-base mutation was found in 12% (n=10) of 84 treatment-experienced patients in the study conducted by He et al.[23]. In our study, primary mutation rtM204I was observed in 2.1% (n=1) and rtL180M alone in 4.2% (n=2). Thus, mutations associated with LAM resistance were seen in 6.3% (n=3) of 48 treatment-experienced cases. Rates of LAM resistance have been reported as 69.7% by Wen et al.[24] in China, 52.9% by Wong et al.[31] in Hong Kong, and 17.3% by Margeridon-Thermet et al.[32] in California. Rates of HBV resistance to LAM in treatment-experienced patients in Turkey were reported as 42.6% by Sayan et al.[33], 28.8% by Timur et al.[8], 22.6% by Bozdayı et al.[27], 19.5% by Alagözlü et al.[34], 15.6% by Aydoğan et al.[26], and 7.6% by Saran et al.[28]. Since LAM is generally used as a first-line therapy, LAM resistance is frequently detected in treatment-experienced chronic HBV patients. The low LAM resistance rate in our study group may be explained by the use of TDF alone or in combined drug regimens. Furthermore, emergence of rtM204V and rtL180M mutations could be affected by many factors such as treatment adherence, dynamics of viral load, efficiency of the viral polymerase, and patients' genetic polymorphisms[35].

In the treatment-experienced group of our study, the coexistence of rtM204V and rtT184S mutations associated with ETV resistance was detected in one patient (2.1%). rtM204V alone without compensatory mutation rtL180M has been documented in some studies[36], but restorative rtL180M mutation may occur later in these cases[37]. The emergence of resistance to ETV in HBV is rare and develops in less than 1% of patients after 4-5 years of treatment. ETV resistance requires LAM mutations (rtM204V and rtL180M) as well as other substitutions in HBV reverse transcriptase such as rtT184, rtS202, or rtM250. Therefore, different ETV resistance substitutions cause varied levels of ETV susceptibility[38,39]. The rtM204V/rtT184S mutation

profile that we detected in one patient probably indicates low sensitivity to ETV. Our finding of 2.1% ETV resistance is higher than the rate of 0.5% for rtM204I+ rtT184A/S/C/G mutation to ETV reported by Hermans et al.[36], but lower than the rates of 4.5% (due to rt184A/S with compensatory mutations) and 5% (4% for rtM204V/I+rtL180M+rtT184A/I/S and 1% for rtM204V+rtL180M+rtS202C) ETV resistance reported by Sayan et al.[40] in two different studies[33].

The prevalence of drug-resistant HBV in antiviral treatment-experienced patients in 18 European countries was reported as 52.7%[36]. Rates of 63%, 25.5%, and 22.6% have been reported in Turkey[26,27,33]. The lower rate of drug resistance (8.3%) seen in our group of treatment-experienced patients might be due to the appropriate selection of patients for treatment and suitable drug combinations. In the treatment-experienced group, 11 (22.9%) of the 48 patients received TDF alone and 12 (25%) received TDF together with LAM ± ADV ± ETV ± IFN. ETV was the first-line treatment in eight (16.6%) patients. LAM, which has low genetic barrier for drug resistance and is not usually recommended as a first-line treatment, was given to only 11 (22.9%) patients.

The pyrosequencing method provides high sensitivity and rapid diagnosis. However, HBV often has spontaneous mutations at low frequency, though these are less clinically significant. Importantly, the frequency of HBV mutations is dramatically increased in patients treated with anti-HBV drugs such as IFN and NAs, particularly in patients with long-term treatment. Pyrosequencing technology has many unique advantages over other DNA sequencing technologies. One advantage is that the order of nucleotide dispensation can be easily programmed and alterations in the pyrogram pattern reveal mutations, deletions, and insertions. Moreover, this technique is carried out in real-time, as nucleotide incorporations and base callings can be observed continuously for each sample[7].

In our study, the low number of samples, single-center design, and inability to follow the patients' treatment regimens prevented a full investigation of the prevalence of HBV drug resistance mutations in our region. Nevertheless, next-generation pyrosequencing has highly sensitive point mutation assays for HBV. Clinical research is needed to determine at what point a minor drug-resistant variant becomes clinically relevant, because HBV polymerase is highly error prone, resulting in one error per 10^4 to 10^5 nucleotides. Therefore, it is only those variants that occur at higher frequencies and in the right mutation contexts that are likely to be clinically meaningful. In addition, the rapid results of the pyrosequencing method may allow detection of antiviral resistance patterns that may occur before and during the treatment. Thus, pyrosequencing may be routinely performed as an HBV drug resistance test[41].

Conclusion

The incidence of drug resistance mutations were 8.3% among treatment-experienced and 1.1% among treatment-naive patients in our study. The relatively low rate of drug resistance seen in treated patients in our study compared to other reports might be due to appropriate selection of treatment and suitable drug combinations. In addition, the use of pyrosequencing technology before and during treatment of patients with chronic hepatitis B infection may facilitate rapid detection of drug resistance mutations. Thus, complications such as cirrhosis and liver cancer due to HBV could be prevented by reducing unnecessary drug use and providing more effective treatment.

Ethics

Ethics Committee Approval: Ethics Committee Approval was received from the Ethics Committee of Çukurova University Faculty of Medicine at the beginning of the study (approval no: 11.10.2013 25/26).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.Ç., F.Y., Concept: F.Y., D.Y., M.Ç., Design: B.Ş., S.K., Data Collection or Processing: S.K., M.Ç., Analysis or Interpretation: M.Ç., F.Y., Literature Search: M.Ç., D.Ö.S., Writing: M.Ç., F.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: Çukurova University Research Funds (BAP SBE TF 2013 YL9).

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