



Molecular Characterization of Hepatitis B Virus Strains Isolated from Chronic Hepatitis B Patients in Southeastern Region of Turkey

Türkiye'nin Güneydoğu Bölgesi'ndeki Kronik Hepatit B Hastalarından İzole Edilen Hepatit B Virüsü Suşlarının Moleküler Karakterizasyonu

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ABSTRACT

Objectives: The aim of the present study was to assess the molecular aspects of HBV strains isolated from chronic hepatitis B (CHB) patients, in the Southeastern region of Turkey.

Materials and Methods: The study involved a total of 110 patients, 57 of them were treatment naive. 53 were undergoing nucleos(t)ide analogue (NUC) therapy, whom were diagnosed with CHB between July 2010 and April 2011 in the Southeastern region of Turkey. We analysed the HBV pol gene by amplification and direct sequencing with using polymerase chain reaction.

Results: The phylogenetic and genotype analysis showed that all (100%) of the patients were infected with HBV genotype D. The prevalence of antiviral drug-associated potential vaccine-escape mutant was 10.5% among treatment naive and 15% NUC therapy group. S gene mutation among treatment naive group and NUC therapy group were 19% and 26.4%.

Conclusion: Determination of genotypes/subgenotypes of HBV may provide robust epidemiological data related to their circulation as well as their transmissibility. However, the findings of HBV pol gene mutations may be helpful in the management of rescue strategies in NUCs resistant patients in Southeastern region of Turkey.

Keywords: Hepatitis B virus, HBV polymerase gene mutation, Nucleos(t)ide analogue

ÖZ

Amaç: Türkiye'deki hepatit B enfeksiyonunun prevalansı Güneydoğu Bölgesi'nde diğer bölgelere göre daha yüksektir. Hepatit B genomundaki mutasyonlar kronik hepatit B hastalarının tanı ve tedavisinde güçlükler sebep olmaktadır. Bu çalışmanın amacı, yüksek endemisiteye neden olan sebeplerinin ve belirli bir suşun bölgede yaygın olup olmadığının değerlendirilmesidir.

Gereç ve Yöntemler: Haziran 2010 ve Nisan 2011 tarihleri arasında Türkiye'nin Güneydoğu Bölgesi'nde kronik hepatit B tanısı alan toplam 110 hasta çalışmaya katıldı. Hastaların 57'si tedavi deneyimsiz iken 53'ü nükleosid analog (NUC) tedavisi almaktaydı.

HBV pol geni amplifikasyon ve direct sekanslama polimeraz zincir reaksiyonu ile analiz edildi.

Bulgular: HBV virusunun filogenetik ve genotip analizi sonucunda hastaların hepsinin (%100) HBV genotip D ile enfekte olduğu görüldü. Antiviral ilaç ilişkili potansiyel aşı kaçak mutantlarının prevalansının tedavi deneyimsiz grupta %10,5 NUC tedavisi alan grupta %15 olduğu bulundu. S gen mutasyonu tedavi deneyimsiz grupta ve NUC tedavisi alan grupta sırasıyla %19 ve %26,4 olarak saptandı.

Sonuç: HBV'nin genotip/subgenotip tayini, virusun yaygınlığı ve bulaşıcılığı hakkında güçlü epidemiyolojik veriler sağlayabilir. Bununla birlikte HBV pol gen mutasyonları ile ilgili bu bulgular, Türkiye'nin Güneydoğu bölgesindeki NUC dirençli hepatit B hastalarında tedavi stratejilerinin yönetiminde yararlı olabilir.

Anahtar Kelimeler: Hepatit B virüsü, HBV polimeraz gen mutasyonu, Nucleos(t)ide analogu

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Introduction

Hepatitis B virus (HBV) infection is endemic at an intermediate to high range within Turkey. The regional hepatitis B surface antigen (HBsAg) prevalence is ranging from 2.5% to 9.1% with the higher values concentrated in the Southeastern part of Turkey (1).

The HBV polymerase (*pol*) gene completely overlaps the envelope gene (2). Mutations in and around the major neutralization domain of HBV known as the "a" determinant, may cause HBV reactivation, diagnostic problems, failure in prophylaxis through vaccination or failure in prophylaxis by administering hepatitis B immunoglobulin (3).

The usage of low genetic barrier/potency drugs such as lamivudine (LAM) in medium-to-high HBV prevalence countries, antiviral drug-associated potential vaccine-escape mutant (ADAPVEM), is becoming a growing health concern (4). The public health significance of such mutant *pol*-envelop overlaps, were recently highlighted when the up to then, theoretical concerns about NUC-resistant HBV, potentiating behaviour as a vaccine escape virus was actually observed in chimpanzees. This drug resistant virus strain was genetically fit and stable, however its' altered envelope escapes the anti-HBs neutralization (5). Transmissions of ADAPVEMs are of critical concern in the control of HBV infections. The generally accepted method for the control of the latter is prevention through vaccination. A secondary approach is the prevention of clinical complications of chronic HBV infections through specific and effective oral antiviral treatments. These being significantly more potent, with higher genetic barriers to resistance, compared to LAM, telbivudine or adefovir (ADV) (5,6).

In this study our aim was to identify the molecular aspects of HBV strains isolated from CHB patients in the Southeastern Region of Turkey where the endemicity is particularly high. We have focused our attention to identify some of the reasons for this high endemicity and whether it is due to a particular strain being prevalent in the region.

Materials and Methods

The informed consents (in Turkish) were obtained from all participants before blood sampling. This study was approved by the Ethical Committee Harran University Faculty of Medicine (approval number: 06/10, date: 02.12.2010).

Patients

Between the dates of July 2010 and April 2011, a total of 110 CHB patients, ages between from 5 to 70 years, with a mean age of 32 years and having a 26% male and 74% female ratio, were enrolled to this retrospective study. CHB infection is defined as the persistence of HBsAg ongoing for 6 months from the date of its first detection. In the beginning of the study 53 patients were already undergoing nucleos(t)ide analogue (NUC) therapy and 57 of the patients were treatment naive.

The patient group undergoing NUC-therapy were receiving LAM (18/53), ADV (1/53), entecavir (ETV) (18/53) and tenofovir (TDV) respectively (16/53).

Inclusion criteria:

- CHB infection with hepatitis B e antigen (HBeAg)-positive or negative

- Treatment naive CHB patients: No previous treatment with Interferon-alpha or NUC,
- CHB Patients already on NUC treatment,
- CHB Patients with compensated liver functions.

Exclusion criteria:

- Co-infection with hepatitis C, hepatitis D, or the human immunodeficiency virus;

- The presence of other forms of liver disease.

Procedures;

The liver damage was classified with Knodell et al. (7) and scaled from 0 to 18 by the histology activity index. Blood samples were separated by centrifugation and the serum was stored at -20 °C until testing. The respective aspartate aminotransferase and alanine aminotransferase levels were measured in the serum by spectrophotometric analysis using standard diagnostic kits (Roche Diagnostics, Mannheim, Germany).

Serological markers of HBV (HBsAg), HBeAg, and antibodies to HBeAg were tested using commercially available micro particle enzyme immunoassay kits, (AxSYM, Abbott Laboratories, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany).

HBV-DNA Detection

The HBV-DNA was isolated from the serum sample using the bio-robot workstation, with magnetic-particle technology (QIA symphony SP, Qiagen GmbH, Hilden, Germany). HBV-DNA was detected and quantified by polymerase chain reaction (PCR) assay (artus HBV QS-RGQ test, Qiagen GmbH, Hilden, Germany) on the real-time platform (Rotor-gene Q, Qiagen GmbH, Hilden, Germany).

HBV Sequencing

A pair of primers (forward: 5'-TCGTG GTGGACTTCTCAATT-3' and reverse: 5'-CGTTGACAGACTTCCAATCAAT-3') were used for the amplification of the HBV *pol* gene region. The up mentioned *pol* gene sequence was already being utilized in our laboratory, for routine HBV genotyping and genotypic resistance analysis). The PCR conditions were determined as in preceeding study (8).

The Determination of HBV Genotypes and Pol/Surface Gene Mutations

We used a phylogenetic analysis and genotyping tool (Geno2pheno) which accepts nucleic acid sequences for the determination of the HBV genotypes as input. The Geno2pheno has a database that is specifically designed for rapid computer-assisted virtual phenotyping of HBV, (Centre of Advanced European Studies and Research, Bonn, Germany, <http://coreceptor.bioinf.mpi-inf.mpg.de/>).

The Geno2pheno searches for homology between the input sequences and others already stored in its database. Additionally stores relevant clinical data for HBV genotypes, drug resistance and S-gene mutations. The tool also searches for HBV drug resistance mutations in the rt domain of the *pol* gene (9).

The genotypic resistance mutations to the NUCs have been categorized as primary or compensatory (3). In our study the overlapping S-gene segment of HBV strain was searched by Geno2pheno and in parallel was checked against previously recorded ADAPVEM HBsAg amino acid substitutions

within its database (10). Some mutations especially ADAPVEMs which were not located in the "a" determinant of the HBsAg protein were observed. The other major neutralising domains of the HBsAg proteins were analysed.

Statistical Analysis

Data entries, determining the mean and the median of different parameters and other preliminary calculations were done on Microsoft Excel. There is no comparison since the prevalence and frequency of the data were given in our study. Genomic values based on bioinformatics were evaluated using bioinformatics-based genotypic rules Geno2Pheno (SVMs) (Centre of Advanced European Studies and Research, Bonn, Germany). Our study does not include statistical significance.

Results

The Gheno2pheno identified that all (100%) of the patients were infected with the HBV genotype D. Among these, the 96.4% of the patients were infected with the sub genotype D1 in the phylogenetic tree. The remaining, 2.7% and 0.9% of the strains were identified as the sub genotypes D2 and D3 respectively (Table 1).

Compensatory (23%) and primary drug-resistance mutations (7%), ADAPVEM (10.5%) and S-gene mutations (11%) were detected in 34/57 (60%) among treatment naive group (Table 2). The ratio of these mutations among patients with viral breakthrough under NUC therapy were 28%, 16.6%, 16.6% and 0% in 18 LAM group, 5.5%, 5.5%, 11%, 22% in 18 ETV group, 0%, 0%, 0% and 100% in ADV group and 0%, 12.5%, 18.7%, 43.7% in TDV group, respectively (Table 3). Six different motifs of ADAPVEM were detected among the CHB patients: rtM250R/sW172L, rtT184G/sL176V, rtM204I/sW196L, rtM204I/sW196S, rtM204V/sI195M, and rtA181V/sL173F. The frequency of ADAPVEM was 12.7% (14/110) in the total CHB patients. The prevalence of S-gene mutation among treatment naive group and NUC therapy group were 19% (11/57) and 26.4% (14/53) respectively (Table 2,3).

Discussion

Eight different genotypes (A-H) of the HBV genome are endemic in different regions of the world (11). The genotype D is prevalent around the Mediterranean Region, the Middle East, and India (12). Sheldon and Co showed that, the mutations in the HBsAg

among LAM treated patients, were higher in the HBV genotype A compared to the HBV genotype D (3). Previous studies have identified the genotype D as being the dominant genotype among CHB patients in Turkey (13). We have similarly observed that all (100%) of our patients were infected with the HBV genotype D.

Replication defects in HBV caused by mutations under the NUC therapy, can be partially repaired by compensatory mutations (3). In our study the prevalence of compensatory mutations among untreated patients were 23%. The mutation patterns are listed as L91I, Q149K, I169V/X, V191F, and Q215H/P/S. The prevalence of compensatory mutations among NUC treated patients were 28% in the LAM treated group. The mutation patterns were L91I or N139K or Q215P or N238T. A sole mutation pattern (A194X) was observed in ETV treated patients and the prevalence was measured as 5.5%. The rtQ215H/Q/P/S compensatory mutations are frequently detected both in treatment naive and NUC treated patients (8,13). We, as well, have detected the rtQ215H/Q/P/S mutations both in treatment naive and LAM treated groups. In a previous paper, the rtL180M mutation was found to be the most common compensatory mutation in a general study among Turkish patients (14). Interestingly, in our study we weren't able to detect any rtL180M mutation.

In our study the prevalence of rt gene mutations among untreated patients was observed as 7%. The mutation patterns were V173M or A181P or I233V or M250R. The same mutation patterns were observed both in the LAM (L80I + rtL180M + rtM204I, T184E/G, M204I) and the ETV (L180M + T184V + M204V) treated patients. However the frequency was higher in the LAM (16.6%) treated patients compared to ETV (5.5%) treated patients. RtL180M + rtM204I, mutations were found among LAM treated patients in a previous study as well. Differing from our results, in this same previous study rt A181V and rt Q215S had also been observed (15).

Some of the mutations in the polymerase gene of HBV are associated with alterations in the "a" determinant of the HBsAg protein. These mutations change the antigenicity of the HBsAg. Such changes may reduce the efficiency of the antibodies induced by the recombinant vaccine (16). In our study we have identified that the vaccine escape HBsAg mutations consist of F161L/H, S193L, M250R/W172, T184G/L176V, M204I/W196L, M204I/W196S, M204V/I195M, M204V/I195M, A181V/L173F or W172L. The prevalence of ADAPVEM was observed to be 10.5% among untreated patients while it was 16.6% with LAM, 11% with ETV, and 18.7% with TDF treatment. Compared to those found in another study held in the northwest region of Turkey, the frequencies of ADAPVEM among untreated and treated patients were found to be higher in our study (15). Additionally, the mutation patterns of the ETV treated patients in our study were different from another previous study. The latter study identified rt I169T, rtT184C, rtT184L/S, rtT184G/M, rtS202C/G and rt S202I mutations. Interestingly, our study identified completely different M204V/I195M, S193L mutation patterns (17). The TDF treated ADAPVEMs were M204V/I195M, A181V/L173F, W172L in our patients. Both studies had only one matching mutation, which was identified as rtA181V (17).

Table 1. Clinical and laboratory characteristics of the study population

Male %, Female %	26%, 74%
Mean age, (range)	32 (5-70)
ALT, median IU/L (range)	81 (23-367)
AST, median IU/L (range)	55 (16-298)
HBV-DNA, median copies/mL (range)	1.2+E9 (2+E4 - 3.2+E10)
HBV genotype (%)	D (100)
- Sub genotype (%)	- D1 (96.4)
	- D2 (2.7)
	- D3 (0.9)
ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, HBV: Hepatitis B virus	

Table 2. Hepatitis B virus pol gene mutation patterns and frequencies among treatment naïve chronic hepatitis B patients

Treatment naïve group (n=57)	Compensatory mutation	Primary drug resistance mutation	ADAPVEM	S-gene mutation
	L91I Q149K I169V/X V191F Q215H/P/S 13 (23%)	V173M A181P I233V M250R 4 (7%)	F161L/H* S193L* M250R/W172L 6 (10.5%)	Q101R I110L T118A G119I P120S P127T G130R S132S/Y T140I S143L D144E 11 (19%)

ADAPVEM: Antiviral drug-associated potential vaccine-escape mutant

Table 3. Hepatitis B virus pol gene mutation patterns and frequencies among nucleos(t)ide analogue treated chronic hepatitis B patients

NUC treated (n=53)	Compensatory mutation	Primary drug resistance mutation	ADAPVEM	S-gene mutation
Lamivudine (n=18)	L91I N139K Q215P N238T 5 (28%)	L80I + L180M + M204I T184E/G M204I 3 (16.6%)	T184G/L176V M204I/W196L M204I/W196S 3 (16.6%)	Q101R Y134F 2 (11%)
Entecavir (n=18)	A194X 1 (5.5%)	L180M + T184V + M204V 1 (5.5%)	M204V/I195M S193L* 2 (11%)	T118A P127T M133I Y134F S143L 4 (22%)
Adefovir (n=1)	-	-	-	S132F 1 (100%)
Tenofovir (n=16)	-	L180M + S202G + M204V A181V + N236T 2 (12.5%)	M204V/I195M A181V/L173F W172L* 3 (18.7%)	P120S P127T T131I Y134*/H 7 (43.7%)

*Naturally present ADAPVEMs; F161L/H, W172L and S193L,
NUC: Nucleos(t)ide analogue, ADAPVEM: Antiviral drug-associated potential vaccine-escape mutant

Study Limitations

The number of the subjects could have been increased.

Conclusion

In this study we evaluated the mutations involving the polymerase/surface gene sequence changes in HBV patients with pre-existing, naturally occurring or with undergoing NUC treatments.

These findings are important to determine the prevalence and type of developing variants to NUCs. Further studies are needed to understand the clinical significance of these polymerase/surface gene sequence changes. We strongly suggest that, every patient who has been diagnosed with CHB, should be checked for the baseline polymerase/surface gene sequence changes, before initiating treatment. This report on the molecular characterization of HBV is the first of

its kind within the Southeastern Region of Turkey. We wish that the results of our study will contribute to the decision-making processes and the choice of the treatment in the future.

Ethics

Ethics Committee Approval: This study was approved by the Ethical Committee Harran University Faculty of Medicine (approval number: 06/10, date: 02.12.2010).

Informed Consent: The informed consents (in Turkish) were obtained from all participants before blood sampling.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.T., M.S., Concept: S.A., S.T., Design: M.A., S.A., Data Collection or Processing: S.T., M.S., Analysis or Interpretation: M.S., M.A., Literature Search: M.A., Writing: M.A.

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