Journal of Viral Hepatitis, 2010, **17**, 23–27 doi:10.1111/j.1365-2893.2009.01149.x

Naturally occurring amino–acid substitutions to nucleos(t)ide analogues in treatment naive Turkish patients with chronic hepatitis B

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Received January 2009; accepted for publication March 2009

SUMMARY. Naturally occurring amino-acid substitutions in the hepatitis B virus (HBV) polymerase gene may be responsible for resistance to nucleoside/nucleotide (NUCs) analogues. To date, only pre-existing lamivudine resistance has been extensively studied. The aim of the present study was to determine the naturally occurring or pre-existing amino-acid substitutions related to NUCs in treatment naive Turkish patients with chronic hepatitis B (CHB). The investigation involved a total of 88 patients (65 males and 23 females; mean age, 34 years; range, 15–61 years) who were diagnosed with CHB between April 2008 and January 2009. According to HBeAg status, 66 patients were HBeAg-negative and 22 patients were HBeAg positive. Naturally occurring substitutions in the HBV polymerase region were detected by DNA sequencing in 17 (19%) and 30 (34%) patients, based on manual and geno2pheno tool database interpretation, respectively. Each amino-acid substitution appeared alone and included rtA194T, rtV214A, rtQ215S, rtI233V and rtN236T. The median values for viral load, ALT and AST were 3.3 $\log_{10}$ (2.0–6.0) IU/mL, 36 (12–515) U/L and 27 (13–284) U/L, respectively, but these did not correlate with the observed amino-acid substitutions in the polymerase region. By direct sequencing, genotype D of HBV was found to still be dominant among Turkish patients. In conclusion, every patient who is diagnosed with CHB should be monitored before the start of treatment for more effective management of patient treatment options.

Keywords: direct sequencing, hepatitis B virus, naturally occurring substitutions, nucleoside/nucleotide analogues.

INTRODUCTION

Two different types of drugs can be used in the treatment of chronic hepatitis B (CHB): interferon alpha and nucleoside/nucleotide (NUCs) analogues. NUCs for hepatitis B virus (HBV) therapy belong to three classes: L-nucleosides (lamivudine, telbivudine and emtricitabine), deoxyguanosine analogues (entecavir) and acyclic nucleoside phosphonates (adefovir and tenofovir). Lamivudine, telbivudine, entecavir, adefovir and tenofovir have been approved in the Europe, the United States, and most Asian and Latin American countries for HBV treatment [1–3].

A major concern with NUC treatment is the selection of antiviral–resistant mutations [4,5]. Mutations selected under NUCs can be split into two groups; those that cause resistance that sometimes leads to a decreased viral fitness, and compensatory mutations, which partially or fully restore the level of viral fitness [6,7]. Some reports have indicated that YIDD and YVDD HBV-drug resistant mutants can be detected in infected individuals prior to treatment with lamivudine [8–10]. To date, only pre-existing lamivudine resistance mutations have been extensively studied [4]. However, naturally occurring amino-acid substitutions related to other NUC have not been investigated in treatment naive patients with CHB. Should reverse transcriptase (rt) amino-acid substitutions occur in some patients with CHB before treatment, their identification would help in making the right decision about the NUC choice and thus avoid unnecessary treatment.

Genotypic resistance assays use DNA sequencing methods to examine the polymerase region of the HBV genome for recognizable resistance-associated mutations [11–13]. Sequence analysis is considered as the gold standard for characterizing HBV DNA isolates [14,15]. However, this assay is time-consuming for a large number of clinical samples, but is suitable for screening of a large region of the viral genome.

Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; NUCs, nucleoside/nucleotide; rt, reverse transcriptase.

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The aim of the present study was to determine the naturally occurring amino-acid substitutions related to NUCs in treatment naive Turkish patients with CHB.

MATERIALS AND METHODS

Patients

The study was carried out between April 2008 and January 2009 at the Kocaeli University Hospital. All of the patients were categorized as HBV chronic carriers according to EASL clinical practice guidelines and were treatment naïve [1]. Blood samples were separated by centrifugation immediately, aliquoted and then kept at −20 °C until testing. Serological markers of HBV were tested using commercially available microparticle enzyme immunoassay kits (Axsym; Abbott Laboratories, Abbott Park, IL, USA and Elecsys, Roche Diagnostics, Mannheim, Germany).

DNA isolation and real-time PCR

HBV DNA was isolated from serum samples by a biorobot workstation using magnetic-particle technology (NucliSENS – easyMAG, bioMérieux, Boxtel, the Netherlands). HBV DNA was detected and quantified by a commercial real-time PCR assay (Iontek Biyotechnology Inc., İstanbul, Turkey, iCycler iQ5, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Sequencing of HBV polymerase gene region

Briefly, a pair of primers was designed (forward: 5′-TCGTG GTGGACTTCTCTCAATT-3′ and reverse: 5′-CGTTGACAGAC TTTCCAATCAAT-3′) for amplification of the HBV polymerase region. The PCR conditions were: 95 °C for 15 min, and then 45 cycles consisting of 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s. The final concentration of the primers was 0.3 μM. The size of the HBV amplicon was 742-bp. All PCR products were purified using the High Pure PCR Product Purification Kit® (Roche Diagnostics GmbH, Mannheim, Germany) and directly sequenced with the ABI PRISM 310 Genetic Analyzer® equipment using the DYEnamic ET Terminator Cycle Sequencing Kit® (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). For the cycle sequencing the following thermal protocol was used: 35 cycles consisting of 95 °C for 20 s, 50 °C for 25 s, and finally 60 °C for 2 min. The reverse primer was used as the sequencing primer at a final concentration of 0.5 μM. The electropherogram-obtained sequences were assembled using Vector NTI® v5.1 (InforMax™ Invitrogen™ Life Science Software, Frederick, MD, USA).

Determination of HBV genotype

HBV genotypes were determined by the genotyping tool of the National Center for Biotechnology Information (NCBI, US National Library of Medicine, Bethesda, USA, http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) that identifies the genotype based on the viral nucleotide sequences. The genotyping tool works by using BLAST to compare a query sequence to a set of reference sequences for known genotypes [16].

Determination of polymerase and surface gene mutations

The Genafor/Arevir-geno2pheno drug resistance tool (Center of Advanced European Studies and Research, Bonn, Germany, http://coreceptor.bioinf.mpi-inf.mpg.de/) for HBV is a database that is specifically designed for rapid computer-assisted virtual phenotyping of HBV, and accepts genome (nucleic acid) sequences as input. Geno2pheno searches for homology between input sequences and others already stored in its database, which also stores relevant clinical data for drug resistance and surface (S) gene mutations. The data accumulated by direct sequencing were analyzed either manually or using the geno2pheno tool. The Geno2pheno tool searches for HBV drug resistance mutations in the rt domain of the polymerase at amino-acid positions 80, 169, 173, 180, 181, 184, 194, 202, 204, 215, 233, 236 and 250. However, rt amino-acid substitutions at positions 84, 85, 214, 237 and 238 were searched for in additional manually [4]. The overlapping S-gene segment was searched by the geno2pheno tool for five amino-acid substitutions at positions 137, 141, 144, 145 and 147. Additionally, this region was also searched for mutations at positions 121, 135, 139, 140, 142, 146, 148, 149, 151, 152, 153, 155, 156 and 157 manually [17].

RESULTS

The study patients consisted of 65 males and 23 females with a mean age of about 34 years (age range 15–61 years). Serological data showed that all patients were hepatitis B surface antigen (HBsAg) positive and HCV, HDV and HIV negative. According to their HBeAg status, 66 (75%) patients were HBeAg-negative and 22 (25%) HBeAg positive. The clinical status of the patients according to the EASL Clinical Practice Guidelines is shown in Table 1.

Naturally occurring rt amino-acid substitutions identified manually and using the geno2pheno tool database were detected in 17 (19%) and 30 (34%) of the patients, respectively. Each amino-acid substitution appeared alone in the patients and included the rtA194T, rtV214A, rtQ215S, rtI233V and rtN236T substitutions (Table 2). In addition, some of the patients had atypical substitutions such as rtM204K/R/T, rtV214E/T and rtI233L/S/T.

Some of the rt amino-acid substitutions such as rtA194T (in two patients) and rtN236T (in one patient) were relevant resistance mutations for tenofovir and adefovir, respectively. The rtA194T mutation was detected in HBeAg-negative CHB patients and the rtN236T in an HBeAg positive patient.

Table 1 Clinical and laboratory characteristics of the study population

Patients (*n*)	88
Male *n* (%)	65 (73.8)
Age, median years (range)	34 (15–61)
HBeAg positive *n* (%)	22 (25)
ALT, median IU/L (range)	36 (12–515)
AST, median IU/L (range)	27 (13–284)
HBV DNA, median log IU/mL (range)	3.3 $\log_{10}$ (2.0–6.0)
HBV genotype (%)	D (100)
Clinical status	Patients newly diagnosed: 38 Patients with Knodel fibrosis scores: 20 Patients awaiting biopsy: 13 Patients in the immunetolerant phase: 12 Patients in waiting period for 6 months: 3 Patients under antiviral treatment: 2

The median viral load, ALT and AST values were 3.3 $\log_{10}$ (2.0–6.0) IU/mL, 36 (12–515) U/L and 27 (13–284) U/L, respectively, and these values did not correlate with the observed rt amino-acid substitutions. In addition, patient age did not correlate either.

The Knodell fibrosis scores of the patients (only for 20 patients in total) were one in 10%, two in 55%, three in 10%, four in 20% and seven in 5%. These did not correlate with the presence of the mutations either.

The direct sequencing results revealed the presence of genotype D in all patients. Also, one of the patients (with rtA194T substitution) had amino-acid changes in the overlapping S-gene segment as sG145R and sC137L together.

DISCUSSION

The ability of viruses to generate resistant variants is facilitated to a great extent by continued viral replication in the presence of an inhibitor [6,18]. In contrast, HBV variants are defined as naturally occurring subspecies that are present in the patients independent of external selection pressures such as drug treatment [7]. Especially with lamivudine-resistant mutants have been reported among patients not treated with the drug with an incidence ranging from 1 to 27% [8–10,19,20]. There is a lack of data regarding the clinical relevance of the naturally occurring rt amino-acid substitutions at the related lamivudine resistant region and other parts of the polymerase gene [10]. In addition, follow-up studies are limited in hepatitis B patients with naturally occurring lamivudine resistance variants and their impact on subsequent treatment [10]. The only related report in the literature is of a patient who had clones of the YIDD mutation only during the pre-treatment period and who then developed severe breakthrough hepatitis after receiving lamivudine [19]. Because drug resistant strains are important in deciding treatment strategy, we explored the rate of rt amino-acid substitutions among untreated patients. The results of the direct sequencing revealed rt amino-acid substitutions among untreated Turkish patients.

Table 2 Emerging amino-acid changes in the HBV polymerase gene in treatment naive patients

		Amino acid substitution profile†				
Patient no.	Method	rtA194	rtV214	rtQ215	rtI233	rtN236
2	Manual/Database	T				
2	Manual		A			
1	Manual		E			
1	Manual		T			
1	Manual/Database			A		
1	Manual/Database			H		
1	Manual/Database			P		
7	Manual/Database			S		
7	Database				L	
6	Database				S	
5	Database				T	
1	Database				V	
1	Manual/Database					T

†*Amino acid abbreviations*: A, alanine; M, methionine; V, valine; Q, glutamine; I, isoleucine; N, asparagines; T, threonine; K, lysine; R, arginine; E, glutamic acid; H, histidine; P, proline; S, serine; L, leucine.

Emergence of natural mutations should be expected due to the HBV genome characteristics. The major causes of drug resistance include viral factors such as the kinetics of viral production and clearance, lack of proofreading capacity during the reverse transcription step which creates a large HBV quasispecies pool and the replication fitness of the latter [21]. The present study demonstrates that naturally occurring mutations are of clinical significance in untreated patients infected with genotype D. The frequency of such mutations in the polymerase gene of HBV is higher (according to database results) than previously reported but their locations are totally different from other studies [8–10,19,20]. Some of these rt amino-acid substitutions such as rtA194T and rtN236T are relevant resistance mutations for tenofovir and adefovir, respectively, according to the EASL clinical practice guidelines [1]. As a result of the wide use and long-term duration of therapy with NUCs, some patients are expected to develop resistance mutations. In our study, we did not detect any of the known rt amino-acid substitutions related to lamivudine resistance. Our opinion on the lack of detection of such lamivudine resistant strains may relate to the direct sequencing approach employed here. The ability to detect resistance variants by direct sequencing is increased for variants representing more than 25% of the viral population [22]. Several independent studies have established that mutations such as rt M204I/V, which are necessary for drug resistance, also reduce viral replication efficiency [23–25]. The replication defects can partially be compensated for by selection of secondary (compensatory) mutations [6,26]. The detected rt amino-acid substitutions in this study were associated with lamivudine (rtQ215S), adefovir (rtV214A, rtQ215S, rtI233V) and tenofovir (rtV214A, rtQ215S), and may function as compensatory mutations [4,7]. In this study, there was no close relation between rt amino-acid substitutions and viral loads of HBV in the patients studied. Viral load may be an indirect marker of replication efficiency. Nevertheless, detailed evaluation of the replication capacity was not one of the aims of this study.

NUC resistant HBV variants can commonly be detected by direct sequencing of HBV DNA [14,27]. However, this assay is time-consuming for a large number of clinical samples, but is suitable for screening of a large region of the viral genome. Phenotyping of HBV mutations using software such as the geno2pheno tool is a convenient approach. Large regions of sequence data can be rapidly analyzed for amino-acid substitutions. In this study, we also manually analyzed the amino-acid sequence between positions 80 and 250 of the HBV polymerase gene. The results were similar between the manual and geno2pheno tool analysis, except at positions rtV214 and rtI233. The cause of dissimilarity for the rtV214 was the exclusion of this position from the analysis carried out by the geno2pheno tool. There is not sufficient data available for the interpretation of the substitution at amino acid position 233. A published report described three patients infected with a rtI233V variant [11]. One of our patients had this substitution also. The functional significance of this mutation is unknown, but a mutation nearby (rtN236T) is associated with adefovir resistance [11].

Several studies have shown that genotype D of HBV represents almost all isolates from the Turkish patient population infected with HBV [10,28–30]. The present study showed that genotype D of HBV is still dominant among Turkish HBV-infected patients. It was not possible to make a comparison relating to different HBV genotypes as the study was conducted in genotype D dominant group. Studies of NUC therapies have not shown any relation between HBV genotypes and response (1). But, it would be worthwhile to see if pre-treatment rt amino-acid substitutions related to NUCs occur more frequently in HBV infections with some genotypes than others.

Because of the overlap of the open reading frames of the HBV polymerase with that of HBsAg, drug resistant mutations in the HBV polymerase can directly impact on the nature of HBsAg and its function [26,31]. Mutations in and around the major neutralization domain of HBV known as the 'a' determinant may result in decreased affinity of HBsAg to anti-HBs and cause diagnostic problems and/or failure of infection prevention with vaccination or HB immunoglobulin [6,7,31–33]. The present study demonstrated that the overlapping S-gene segment was affected by pre-existing rt amino-acid substitutions. Also, one of the patients (with rtA194T substitution) had amino-acid changes in the S-gene (sG145R and sC137L). Mutant sG145R is the most frequently reported and is known as a vaccine escape mutant [6,14,33]. It appears to impair seriously the performance of many commercial tests, and is responsible for false-negative results [17]. However, it was picked up as positive by the Axsym and Elecsys tests used in this study.

In conclusion, we detected various naturally occurring rt sequence changes related to NUCs in treatment naïve Turkish patients with CHB. The pre-existing or naturally occurring rt sequence changes may be important to elucidate the prevalence and type of developing variants to NUCs. In addition, prospective studies are needed to define the clinical significance of these baseline rt sequence changes. Also every patient who is diagnosed with CHB should be monitored for the baseline rt sequence changes before initiating treatment for a more effective treatment outcome.

ACKNOWLEDGEMENT

We thank Onur Bilenoğlu (from Iontek Biyotechnology Inc, Istanbul, Turkey) for support with the direct sequencing studies.

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