

## Ultra-Deep Pyrosequencing of Precore/Core Promoter Mutations in Patients with Genotype D Chronic Hepatitis B

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Although G1862A mutation was common in HBeAg-negative patients in our study, this mutation was positive in 1 HBeAg-negative and 6 HBeAg-positive patients. The G1896A precore stop codon mutation rate was 33% in HBeAg-positive and 75% in HBeAg-negative patients. The total detectable precore and BCP mutation rates were 92%, and 67%, respectively.

**CONCLUSION:** Precore/core promoter mutations were frequent in patients infected with genotype D HBeAg-positive HBV. These findings show that HBeAg can be positive even in the presence of precore mutations.

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**Key words:** Precore/core promoter mutations; Genotype D; HBV

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### ABSTRACT

**AIM:** The clinical course is different in patients who are HBeAg-negative or positive during the natural course of chronic hepatitis B virus infection. In this study, we aimed to investigate the prevalence of precore and basal core-promoter mutations in chronic hepatitis B patients using ultra-deep pyrosequencing

**METHODS:** Sixteen patients with treatment naive chronic hepatitis B (12 HBeAg-positive) were studied (8 male, mean age 29.2±12.8 years). Pretreatment serum HBV-DNA concentrations were determined using a Bayer bDNA Analyzer system 340 and UDPS was performed using the GS FLX platform.

**RESULTS:** All patients were genotype D1. A1762T-G1764A core-promoter double mutations were detected in 2(17%) HBeAg-positive patients, but not detected in any HBeAg-negative patients. T1842C mutation was observed in 5 HBeAg-positive patients, but not observed in HBeAg-negative patients. This mutation was accompanied with G1816A and C1845T mutations in each patient.

### INTRODUCTION

Chronic hepatitis B virus (HBV) infection has a wide range of clinical presentations from inactive carriage to cirrhosis. HBV has a dynamic genome which makes it possible to form several variants during chronic infection. The most common naturally occurring HBV variants include the precore stop codon mutation (G1896A), which abolishes hepatitis B e-antigen (HBeAg) production, and the dual mutation in the core promoter region (A1762T, G1764A), which down-regulates HBeAg production<sup>[1-5]</sup>. Chronic HBV infection is a dynamic process. The natural history of chronic HBV infection can be divided into five phases, which are not necessarily sequential: immune tolerant phase (HBeAg positive), immune reactive HBeAg-positive phase (HBeAg-positive, lower level of replication), inactive HBV carrier state (may follow seroconversion from HBeAg to anti-HBe), HBeAg-negative CHB, and hepatitis B surface antigen

(HBsAg) negative phase<sup>[6]</sup>. Precore (38% vs 9%) and basal core promoter (BCP) (51% vs 36%) variants are more common in HBeAg-negative patients compared with HBeAg-positive patients<sup>[5]</sup>. Clinical features of HBeAg-negative and positive chronic hepatitis B are different. The response to treatment also differs in patients who are HBeAg-positive and HBeAg-negative. If antiviral treatment [nucleos(t)ide analogs] is discontinued before HBsAg clearance, relapse usually occurs in HBeAg-negative patients. Relapse following interferon therapy is also common. Relapse rates can be high in HBeAg-positive patients as well. Continuation of lamivudine therapy after achieving HBeAg seroconversion was associated with a higher proportion of undetectable HBV-DNA and a lower incidence of ALT flares, when compared with those with cessation of lamivudine in Asian HBeAg-positive patients<sup>[7,8]</sup>. Precore/core promoter variants probably affect the clinical features of chronic hepatitis B. These variants are most common in genotype D patients<sup>[9]</sup>. Some studies report that precore/core promoter variants are associated with fulminant hepatitis, more active chronic hepatitis and hepatocellular carcinoma<sup>[10-12]</sup>. Recently, next-generation sequencing methods have been developed to investigate drug resistance and viral tropism in depth. One of these methods is the Genome Sequencer FLX/454 Life Science platform<sup>[13]</sup>. This is more advantageous compared with the conventional population-based sequencing and it can detect minor populations of viral variants in addition to the known viral quasispecies. In this study, we aimed to investigate the prevalence of mutations in the core and basal core promoter of HBV genome using ultra-deep pyrosequencing (UDPS).

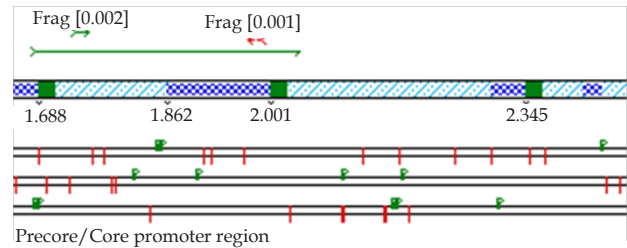
## METHODS

### Patients

Sixteen patients who had chronic HBV (12 HBeAg positive) were enrolled in the study. Eligibility criteria for the study were as follows: (1) Positive HBsAg for at least 12 months; (2) HBV-DNA levels greater than 2000 IU/mL in serum for 6 months or more; (3) elevation (at least  $>1.3\times$  upper limit of normal) of serum alanine aminotransferase (ALT) levels for at least 3 months; and (4) biopsy proven chronic hepatitis. All the other etiologies of chronic hepatitis were excluded in these patients. Antibody to hepatitis C virus (anti-HCV) and total antibody to hepatitis delta virus (anti-delta total) were also negative. A liver biopsy was performed at baseline in all patients. Histologic changes [histology activity index (HAI), and the extent of fibrosis] were assessed according to the Knodell scoring system<sup>[14]</sup>. None of the patients received any treatment prior to the collection of serum samples. The serum HBV-DNA concentrations were determined using Bayer bDNA Analyzer system 340.

### Amplification of the core promoter/pre-core region

Amplification of precore/core and S genes using conventional polymerase chain reaction (PCR) Conserved HBV genomic sequences from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) were chosen to design 10 pairs of overlapping amplicons to detect the precore/core and S genes using PCR primers (Table 1, Figure 1) (IDT, Bradford, CT). Barcoded amplicon primers were designed according to GS-FLX 454 recommendations ([www.454.com](http://www.454.com)). All fragments were amplified using a proofreading enzyme (FastStart High Fidelity enzyme, Roche, Mannheim, Germany). We added 10ng of HBV DNA to the PCR mix, 50 uM dNTPs, 1.5 mM MgCl<sub>2</sub>, 10 pmol primers, 0.15 U Taq polymerase (FastStart High Fidelity enzyme, Roche, Mannheim, Germany). In total, 35 rounds of PCR amplification were performed in order to maximize the amount of viral DNA. PCR was performed as follows: one at 95°C for 3



**Figure 1** An overview for primer localization in precore/core promoter region.

**Table 1** Amplification primers used in the PCR for UDPS of HBV-genome.

Primer No.	Primer Sequences(5'→3')
1	GCCTCCCTCGCGCCATCAGCTCCACCACGTTCCACAAACTC
2	GCCTTGCCAGCCCGCTCAGAAGAAGATGAGGCATAGCAGCAGG
3	GCCTCCCTCGCGCCATCAGCCAAATCTCCAGTCACTCACC
4	GCCTTGCCAGCCCGCTCAGGAATACAGGTGCAGTITCCGTCC
5	GCCTCCCTCGCGCCATCAGAGAACCCTGCACGACTCTGTCTCA
6	GCCTTGCCAGCCCGCTCAGGACATACCTTCCAATCAATAGG
7	GCCTCCCTCGCGCCATCAGCTTGAGTCCCTTTATGCCGCTG
8	GCCTTGCCAGCCCGCTCAGGACAGTATGGATCGGCAGAGG
9	GCCTCCCTCGCGCCATCAGGTGCCAAGTGTTCGTGACCC
10	GCCTTGCCAGCCCGCTCAGGAAGTATGCCTCAAGGTCCGTC

minutes, and 35 cycles comprises; 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, and one final cycle at 72°C for 5 minutes.

### UDPS

PCR products (333 bp long) were purified using QIAspin mini-elute spin kit (QIAGEN Co. USA). PCR amplified DNA were used to generate the amplicon Library according to the manufacturer's protocol (Roche Diagnostics Co., Indianapolis, IN, USA). DNA fragments were cleaned using the Mini-Elute Kit (Qiagen Inc, Valencia, CA, USA) and a fragment size and quality was verified using Agilent Bioanalyzer 2100 (Agilent Technologies, Anaheim, CA, USA). GS FLX runs were performed using the 16-lane gasket picotiter sequencing plate, and GS FLX kits were used to produce UDPS according to the manufacturer's protocol (Roche Diagnostics Co.). AVA (Roche, Mannheim, Germany) and Sequencer (Genes Codes Corporation) software was used to analyze UDPS results. Mutation frequency was estimated using the AVA software with the cutoff set to 5%.

The raw sequences obtained were sorted by barcodes and each individual's sequence reads were determined. The sequence reads were then aligned to the Reference Sequence (AY721608)<sup>[15]</sup>. Single nucleotide variations of each patient were determined according to the following criteria: variants must be detected in at least three unique (non-duplicate) sequencing reads with both forward and reverse reads and high quality scores ( $>Q20$ ) for variants. Some proportions of sequences were rejected because of insufficient quality.

### Statistical Analysis

Statistical analysis (SPSS 15.0 statistical software package) was carried out using Pearson chi-square test, Fisher's exact and correlation test when necessary. *P*-values less than 0.05 were considered statistically significant.

## RESULTS

Sixteen patients who had treatment naïve chronic HBV [12 HBeAg(+)] were studied (mean age 29.2±12.8 years). The patients'

viral loads and histologic findings are shown in table 2. All patients were genotype D1. UDPS for precore/core promoter region was performed using GS FLX platform; 2,731,902 nucleotides and an average of 512 depths were obtained from of all 16 samples.

Precore and basal core promoter mutations are shown in table 3. A1762T-G1764A core-promoter double mutations were detected in 2 (17%) HBeAg-positive patients, but not detected in HBeAg-negative patients. The T1842C mutation was observed in 5 HBeAg-positive patients, but not in HBeAg-negative patients. This mutation was accompanied with C1816A and C1845T mutations in each patient. Although G1862A mutation is common in HBeAg-negative patients, it was positive in 1 HBeAg-negative and 6 HBeAg-positive patients in our study. The G1896A precore stop codon mutation rate was 33% (n=4) in HBeAg-positive and 75% in HBeAg-negative patients. Detectable precore and BCP mutation rates were 92%, 67% in HBeAg-positive and 75%, 50% in HBeAg-negative patients, respectively (Figure 2). There was a negative correlation between

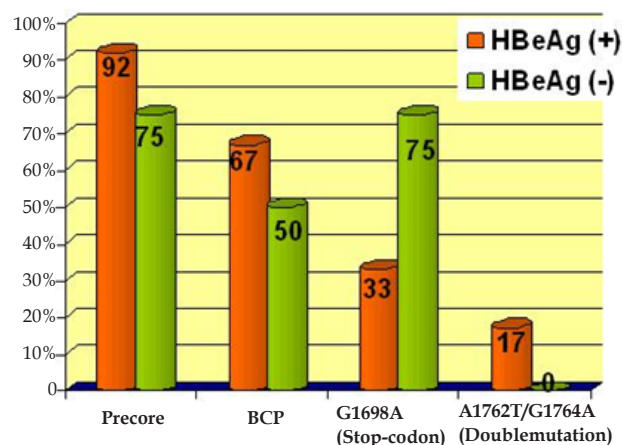


Figure 2 Distribution of precore, BCP mutations rate in HBeAg positive and negative.

	HBeAg (+) N=12	HBeAg (-) N=4
Age, years	26.5 ±12.9	37.5±9.03
Gender (M/F)	5/7	3/1
HAi (mean)	6.3 ±2.4	8.7±3.3
Stage (1/2/3/4)	8/2/0/2	2/0/1/1
Advanced fibrosis	17%	50%
HBV DNA IU/mL(mean)	30,796,563±45,728,458	16,853±85,505
ALT IU/L (mean)	143±86	164±159

	HBeAg positive												HBeAg negative			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Precore																
G1816A					60	25	14		5.8			6		14	19	
T1842C					60	7.6	14		6.5			6.5				
C1845T	11				60	23	60		6.8			6.5		70		
T1846A			30			19				18		30		16		
G1862A			71		17.3				33	14		80	30			10
¥T1885G									4			22	33			22
G1896A					33	17					19	97		31	5	16
G1899A					33	17			33					90	90	
Basal core promoter																
A1752C									5.5			3				
T1753C												3				22
T1354C																30
<sup>1</sup> A1757G	11				15			30	11			8.5		7	50	
A1762T	12.5								27							
G1764A	12.5								27							
<sup>1</sup> C1773T	11	20	5									12			20	

<sup>1</sup> synonym mutation.

fibrosis and presence of a precore mutation ( $P=0.003$ ,  $r=-0.698$ ). Only 2 patients who were precore mutation positive had advanced fibrosis stage (stage 3 and 4). Interestingly, the precore mutation rate was significantly lower in patients aged over 40 years (85.7% vs 14.3%,  $p<0.05$ ) in the total group. There was a negative correlation between age and presence of precore mutations ( $P=0.009$ ,  $r=-0.63$ ). However, we did not find any correlation between HBV-DNA levels and presence of mutations and fibrosis stage.

## DISCUSSION

Precore/core promoter mutations were generally analyzed using direct PCR sequencing in the literature. In this study, we analyzed 16 treatment naïve chronic hepatitis B patients using UDPS. The most important limitation of our study was the number of patients; this was due to technical difficulties. In our study, precore and BCP mutations rates were higher in HBeAg-positive patients than HBeAg-negatives (92%, 67% vs 75%, 50% respectively). Chu *et al*<sup>[15]</sup> reported that these mutations are more common in patients who are HBeAg-negative than patients who are HBeAg-positive (precore, 38% vs 9%; core promoter, 51% vs 36%; respectively,  $P<0.001$ ). In this study, the rate of genotype D patients was 10.4%. Precore variants were more common in genotype D (55%), whereas core promoter variants were more common in genotype C (60%). In Turkey, genotype D is the most common genotype<sup>[15]</sup>. In our study, all patients were also genotype D. Interestingly, the rate of A1762T-G1764A core-promoter double mutations was 17% in HBeAg-positive patients, as compared with none in HBeAg-negatives. Fang *et al*<sup>[16]</sup> showed that BCP double mutations are associated with lower viral load in HBeAg-positive patients. Sonneveld *et al*<sup>[17]</sup> was reported that precore (G1896A) variant rate was 27% and core promoter (A1762T, G1764A) variant rate was 44% in HBeAg positive patients. Chu *et al*<sup>[15]</sup> also reported that HBeAg-positive patients with a selection of core promoter variants have lower serum HBV-DNA levels; whereas HBeAg-negative patients with core promoter or precore variants have higher serum HBV-DNA levels compared with the HBeAg positive patients. We did not find a relationship between HBV-DNA viral load and BCP/precure variants in our study. This may be related to the genotypes or to the limited number of patients. On the other hand, 33% of HBeAg-positive patients had stop codon mutations in our group. The frequency of variants varied from 17% to 97% of the total HBV population (Table 3). Therefore, our study clearly showed that double mutations (A1762,

G1764A) and stop codon mutation (G1896A) do not prevent the synthesis of HBeAg in genotype D patients. Either a precore stop codon or a basal core-promoter mutation was detected in all HBeAg positive patients. Among four HBeAg-negative patients, only one did not have a mutation. Among 5 of the HBeAg-positive patients, the precore stop codon mutation rate made up more than 50% of the viral population. Conversely, the basal core promoter mutation rate was less than 50%. In half of the HBeAg-negative patients, the precore stop codon mutation rate was 90%. These results show that the viral pool is heterogeneous in patients with a chronic HBV infection. Because of the limited number of patients, we cannot make a definite correlation between the detected mutations and clinical, and histologic findings.

HBV replication *in vivo* is characterized by a high turnover and mutation rate. UDPS has an increased sensitivity for detecting low-prevalence variants, including minor drug-resistance mutations at a level of sensitivity, which were previously impossible. With appropriate analysis, UDPS is a promising method for characterizing genetic diversity and detecting minor, yet clinically relevant variants in biological samples with complex genetic populations. UDPS has a more sensitive method to detect viral mutations in comparison with other methods.

Treatment response and prognosis are different between HBeAg-positive and -negative patients. Long-term treatment is recommended for HBeAg-negative patients. However, HBeAg loss or seroconversion in HBeAg-positive patients is an important endpoint, as this is usually associated with sustained remission and there is a low risk for development of cirrhosis and hepatocellular carcinoma<sup>[18-19]</sup>. In most studies, relapse rates were high in HBeAg-positive patients after treatment cessation. Lee *et al.*<sup>[20]</sup> showed that the cumulative relapse rates increased from 15.9% at 1 year to 30.2% at 5 years, with a mean time to relapse of 12 months (range, 7-42) after cessation of lamivudine treatment. Most relapses occurred within 2 years after discontinuation of lamivudine (33/40, 82.5%). In the study of Reijnders *et al.*<sup>[21]</sup>, recurrence after treatment discontinuation or noncompliance was observed in all patients treated with nucleos(t)ide analogues. Therefore, long-term continuation of treatment appears to be necessary. There are few studies about the relationship between the relapse rate and genetic features of HBeAg-positive patients. A recently published study showed that HBeAg-positive patients with detectable PC and/or BCP mutants (by line-probe assay) have a lower probability of response to pegylated interferon treatment<sup>[17]</sup>.

As a conclusion, our study shows that precore/core promoter mutation rates are more frequent in patients infected with HBeAg-positive genotype D HBV and that HBeAg can even be expressed in the presence of precore mutations. Further studies are needed to evaluate treatment response and the rate of relapse in HBeAg-positive patients infected with PC/BCP mutants.

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The Ethics Committee of the Istanbul Faculty of Medicine approved the study protocol (06082010-03), and all participants gave written informed consent for the study. This study was financially supported by the Research Fund of Istanbul University, Turkey (project no. 06082010-03).

## CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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