HLA DPB1 15:01 Allele Predicts Spontaneus Hepatitis B Surface Antigen Seroconversion

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Abstract

Aim : **Chronic hepatitis B (CHB) is a global health problem. Recent genome-wide association studies (GWAS) exposed significant association between the human leukocyte antigen (HLA) class II region, including both DP and DQ loci, and chronic hepatitis B. Previous research also indicated the involvement of adaptive immune system in Hepatitis B seroconversion. The aim of this study is to investigate possible polymorphisms in the HLA-DP locus that can contribute to immune response to Hepatitis B virus (HBV).**

Methods : **We enrolled 94 chronic hepatitis B (CHB) patients and a control group of 85 spontaneous seroconverted healthy subjects and genotyped HLA-DPB1 alleles by polymerase chain reaction followed by restriction length polymorphism (PCR-RFLP) and Sanger sequencing.**

Results : **Among the 19 DPB1 alleles analyzed in this study, DPB1*15:01 allele was more frequent in the spontaneous seroconverted control group compared to CHB patients (15.3% vs. 1.1%, χ^2 = 12.5, OR = 0.06, 95% CI = 0.08-0.046 P < 0.001, Pcorrected < 0.001). DPB1*02:01 and DPB1*10:01 were the other alleles observed more frequently in the control group (38.8% vs. 22.3% P = 0.02 and 16.5% vs. 5.3% P = 0.02, respectively). However associations of these two alleles were lost their significance after Bonferoni's correction (Pcorrected = 0.4 for all).**

Conclusions : **In conclusion, this study demonstrates that HLA alleles may participate in spontaneous HBsAg seroconversion which is the ultimate target in CHB in Turkish CHB patients.** (Acta gastroenterol. belg., **2017**, 80, **351-355**).

Keywords : Chronic hepatitis B (CHB) ; HBsAg seroconversion ; Immune response ; HLA-DP; PCR-RFLP 2

Introduction

Hepatitis B virus (HBV) which poses serious threats to human health, currently affects about 350 million people worldwide (1). Hepatitis B infection which is a leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) and results in approximately 800.000 annual deaths (2). Chronic HBV infection is an immunological driven disease where host immune response, besides viral factors, involve in viral persistency or viral elimination (3-5). An accurate host immune response against the virus requires human leukocyte antigen (HLA)-restricted T lymphocytes, antibody secreting B lymphocytes of the humoral immunity system, dentritic cells, natural killer cells and plentiful cytokines (5-7).

The ultimate target of CHB is seroconversion which means becoming HBsAg negative and developing anti-HBs (8-10). HBsAg seroclearance rate varies between different ethnicities. This difference may be caused by difference in the incident time of HBV infection. In communities with more adolescent infections, such as western countries, the incidence of delayed HBsAg clearance ranges from 1% to 2% per year; while in endemic areas, where HBV infection is primarily acquired perinatally or in early childhood lower rates of HBsAg clearance, from 0.05% to 0.8% per year, are reported (11).

Human leukocyte antigens that are responsible for antigen presentation to T cells are crucial elements of adaptive immunity (12). Recent genome-wide association studies (GWAS) revealed significant associations between HLA class II region (DP and DQ loci) and CHB in Asian populations (13-16). Moreover, the association between the HLA class II alleles and clearance of HBV was demonstrated by the candidate gene approach in African, Caucasian and Asian populations (17-20). Furthermore, our research about CHB has demonstrated that HLA-DQB1*05:01 and DQB1*05:03 alleles are strongly correlated to chronic active disease outcome and treatment non-response, respectively (21, 22). Recent GWAS analysis in Asian CHB patients was also indicated that single nucleotide polymorphisms (SNPs) in HLA-DPA1 and HLA-DPB1 alleles are associated with protection against chronicity and therefore immune response against HBV (13, 23), findings that were validated in following studies (16, 24). In addition to GWAS, another genotyping study in Japanese population showed that genetic variants in the HLA-DP locus are significantly related with risk of persistent infection by HBV demonstrating HLA-DPA1*02:02, DPB1*05:01, DPA1*02:02, DPB1*03:01 as risk types and HLA-DPA1*01:03, DPB1*04:02,DPA1*01:03, DPB1*04:01 as protective alleles for 3 persistent HBV infection, respectively (25). In this study, we aimed to investigate

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the association between HLA-DPB1 alleles and CHB, by comparing HBV infected patient's genotype with spontaneous seroconverted healthy controls.

Methods

Study Participants

In this case control study, a case group of 94 unrelated Turkish asymptomatic HBV carriers were recruited from Department of Gastroenterology, Umraniye Teaching and Research Hospital according to the results of serological tests (liver functional indexes, HBV virological indexes) and symptoms of hepatitis B. Chronic hepatitis B was diagnosed if serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were continuously abnormal, with HBsAg and/or HBeAg seropositive, anti-HBs antibodies (anti-HBs) seronegative. Asymptomatic HBV carriers were identified with the following diagnostic criteria: HBsAg seropositivity lasts for more than 1 year, with normal liver functional indexes and without any signs and symptoms of hepatitis and HBV DNA levels were below 2000 IU. Age and gender matched 85 HBsAg negative, anti-HBs positive and anti –Hbc IgG positive spontaneous seroconverted healthy subjects were enrolled as the control group. Local ethics committee approved the study, and all participants gave informed consent. Patients with delta virus co-infection, hepatitis C virus (HCV) or human immunodeficiency virus (HIV) or other liver disease rather than HBV, patients receiving immunosuppressive treatment, and patients under age 18 were excluded from the study.

HLA-DPB1 Genotyping

Genomic DNA was extracted from 200 µL of peripheral blood by using Invitrogen PureLink genomic DNA purification kit (Life Technologies, CA, USA) according to manufacturer's protocol. The alleles of HLA-DPB1 were genotyped by PCR-RFLP (26). Genomic DNA was amplified by the PCR procedure with 0.5 units of the DreamTaq Green DNA polymerase (Thermo Fisher Scientific), 1mM MgCl2, 0.8 mM dNTPs, 0.2 mM forward and reverse primers. The reaction mixture was subjected to 3 min of initial denaturation at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 50 sec at 72°C, 7 min of final extension at 72°C by automated PCR thermal sequencer. The forward and reverse amplification primers for DPB1 allele were 5' GTGAAGCTTTCCCCGCAGAGAATTAC 3' and 5' 4 CCTGCAGTCACTCACCTCGGCGCTG 3' respectively and yielded a 299 bp product. After amplification, aliquots (7 µL) of the reaction mixture were digested with restriction endonucleases (Bsp1286I, FokI, AvaII, DdeI, BssHII, Cfr13I, RsaI, EcoNI, BsaJI; 5 units) at 37°C for 10 h after addition of appropriate incubaction buffer. When digested with BsaJI, the reaction mixture was incubated at 55°C for 10 h.

Agarose Gel Electrophoresis

Samples of the restriction enzyme-cleaved amplified DNAs were subjected to electrophoresis in 2% agarose gels for Bsp1286I, FokI, DdeI, BssHII, Cfr13I, AvaII digestion and 4% for BsaJI, RsaI, EcoNI digestion in a horizontal midi gel apparatus (CS Cleaver Scientific). Cleavage or no cleavage of amplified fragments was detected by staining with SYBR Green I nucleic acid gel stain (Sigma-Aldrich, MO, USA).

Verification with Sanger Sequencing

Randomly chosen 50 PCR products were also sequenced by Sanger sequencing to verify PCR-RFLP methodology. First, PCR cleanup was performed by enzymatic reactions (Exo I-SAP enzymes) to the samples. Sequencing reactions were prepared with ABI "BigDye Terminator v3.1 Cycle Sequencing Kit" according to manufacturer's protocol. Following sequencing, a sequence cleanup process was performed by Zymo "ZR Sequencing Clean Up Kit" with the directions of kit instructions. Then the prepared samples for sequencing were loaded to the instrument Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, CA, USA) and run with the related protocol of instrument. Samples that completed the sequencing process were analyzed using Sequencing Analysis 5.3.1 software.

Statistical Analysis

The frequencies of HLA alleles were calculated by direct counting. Statistical analyses were done to evaluate difference in HLA-DPB1 alleles between CHB patients and spontaneously seroconverted controls. During HLA analysis, 2x2 tables and chi square test were used. When the sample sizes were small or expected values in cells of chi square table were smaller than 5, Fisher's exact test was performed. Parametric variables were assessed by student's t-test. All *P* values were calculated double sided, and *P* values below 0.05 was considered as statistically significant unless there are multiple comparisons. Bonferoni's correction was applied for multiple comparisons and corrected *P* values (*P*c) were given. All analyses were performed in SPSS 21 software (Chicago, IL). 5

Results

Demographic Data of Study Groups

In this study, a case group of 94 hepatitis B patients and a control group of age and gender matched 85 HBsAg negative, anti-HBs positive and anti–HBc IgG positive spontaneous seroconverted healthy subjects from Department of Gastroenterology, Umraniye Teaching and Research Hospital were enrolled. CHB patient group

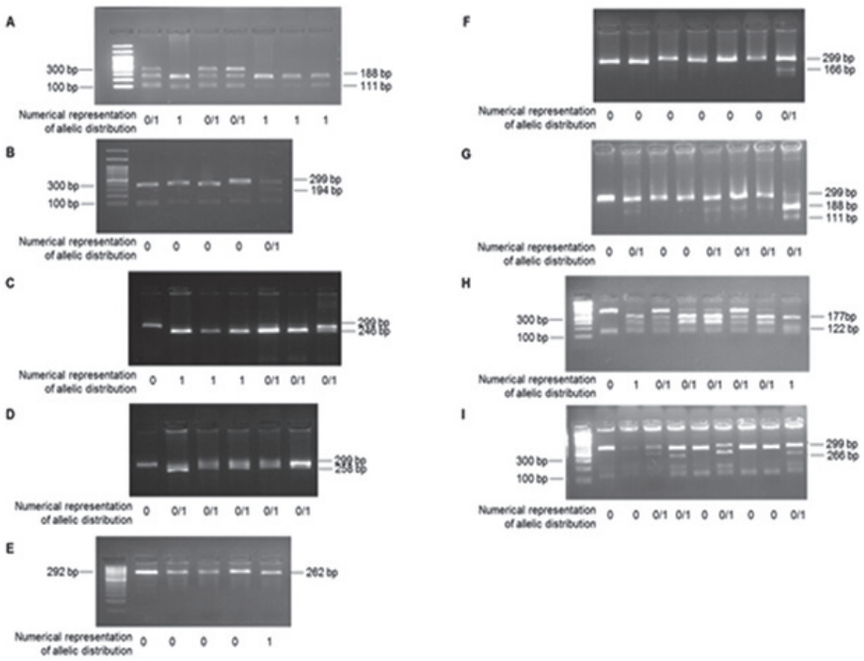

Fig 1. — Agarose gel electrophoresis analysis of RFLP from blood samples. 100 bp DNA ladder used as a marker. Cleaved fragments were marked as "1", non-cleaved fragments were marked as "0", showing these alleles as homozygotes. If the number consists of both cleaved and non-cleaved fragments, this suggests the presence of heterozygotes as "0/1". (A) Cleavage with Fok1. (B) Cleavage with Ava II. (C) Cleavage with BsaJI. (D) Cleavage with Cr13I. (E) Cleavage with Bsp1286I. (F) Cleavage with DdeI. (G) Cleavage with BssHII. (H) Cleavage with RsaI. (I) Cleavage with EcoNI

consists of 58 male (61.7%) and 36 (38.3%) female patients with a mean age 47.9±14.7. Among these patients 71 (75%) of them were HBeAg negative, 16 (17%) had inactive disease. The pretreatment mean ALT,

Table 1. — **Characteristics of patients and control group**

Patient Group (Group A)		**n (%)**
Sex	*Male*	58 (61.7)
	Female	36 (38.3)
HBeAg	*Negative*	71 (75)
	Positive	23 (25)
Cirrhosis	*Yes*	20 (21.3)
	No	74 (78.7)
Control Group (Group B)		n (%)
Sex	*Male*	46 (54.1)
	Female	39 (45.9)
HBsAg	*Negative*	85 (100)
	Positive	0 (0)
Anti HBsAg	*Negative*	0 (0)
	Positive	85 (100)
Anti HBc IgG	*Negative*	0 (0)
	Positive	85 (100)

Tabel 2. — **Frequencies of HLA-DPB1 alleles**

HLA alleles	**Total**		**Hepatitis B Patients**		**Sero-converters**	
	N=179	AF[1]	N=94	AF[2]	N=85	AF[3]
DPB1*04:01	29	0.162	18	0.191	11	0.129
DPB1*04:02	38	0.212	25	0.266	13	0.152
DPB1*02:01	54	0.3	21	0.223	33	0.388
DPB1*18:01	0	0	0	0	0	0
DPB1*02:02	0	0	0	0	0	0
DPB1*08:01	9	0.05	2	0.021	7	0.082
DPB1*05:01	2	0,011	0	0	2	0.023
DPB1*03:01	8	0.045	3	0.032	5	0,058
DPB1*06:01	2	0,011	1	0,011	1	0.011
DPB1*11:01	6	0.034	1	0.011	5	0.058
DPB1*01:01	30	0.167	19	0.2	11	0.129
DPB1*09:01	0	0	0	0	0	0
DPB1*10:01	19	0.1	5	0.053	14	0.164
DPB1*13:01	12	0.067	7	0.074	5	0.058
DPB1*19:01	1	0.006	0	0	1	0.011
DPB1*14:01	13	0.073	6	0.064	7	0.082
DPB1*15:01	14	0.078	1	0.011	13	0.152
DPB1*16:01	30	0.168	20	0.212	10	0.117
DPB1*17:01	17	0.09	10	0.106	7	0.082

1: AF, frequency of all alleles.
2: AF, frequency of Hepatitis B patients (Group A) alleles.
3: AF, frequency of Control Groups (Group B) alleles.

Table 3. — **Distribution of HLA alleles among patients and control group**

HLA Alleles	Hepatitis	Seroconverters	*P*	X^2	OR	95% CI
	Patients (%)	(%)				
DPB1*04:01	18(19.1)	11(12.9)	0.3	1.3	1.6	0.7-3.6
DPB1*04:02	25(26.6)	13(15.3)	0.1	3.4	2.0	0.95-4.24
DPB1*02:01	21(22.3)	33(38.8)	0.0	5.8	0.5	0.24-0.87
DPB1*18:01	0	0	NA	NA	NA	NA
DPB1*02:02	0	0	NA	NA	NA	NA
DPB1*08:01	2(2.1)	7(8.2)	0.1	3.5	0.2	0.05-1.2
DPB1*05:01	0	2(2.4)	0.1	2.2	NA	NA
DPB1*03:01	3(3.2)	5(5.9)	0.4	0.8	0.5	0.12-2.28
DPB1*06:01	1(1.1	1(1.2)	0.9	0.0	0.9	0.56-14.67
DPB1*11:01	1(1.1)	5(5.9)	0.1	3.2	0.2	0.02-1.50
DPB1*01:01	19(20.2)	11(12.9)	0.2	1.7	1.7	0.76-3.83
DPB1*09:01	0	0	NA	NA	NA	NA
DPB1*10:01	5(5.3)	14(16.5)	0.0	5.9	0.3	0.098-0.829
DPB1*13:01	7(7.4)	5(5.9)	0.7	0.2	1.3	0.39-4.22
DPB1*19:01	0	1(1.2)	0.3	1.1	NA	NA
DPB1*14:01	6(6.4)	7(8.2)	0.6	0.2	0.8	0.25-2.36
DPB1*15:01	1(1.1)	13(15.3)	**<0.001**	12.5	0.1	0.01-0.47
DPB1*16:01	20(21.3)	10(11.8)	0.1	2.9	2.0	0.89-4.62
DPB1*17:01	10(10.6)	7(8.2)	0.6	0.3	1.3	0.48-3.66

Based on χ^2 test. $P \leq 0.05$ are shown in bold; OR. odds ratio; 95% CI. confidence interval NA: Not applicable

AST and log DNA were 102.1±113 U/L, 70±96 U/L and 4.9±2.8 IU/ml, respectively. Among them 54 (57.4%) had liver biopsy; the mean Ishak fibrosis score was 2±1.5 and hepatic activity index was 6.5±3.3. Twenty (21.3%) patients had cirrhosis. The mean follow up time for the patients was 76±52 months. Control group formed by healthy people who had already HBV infection with spontaneous HBsAg seroconversion (HBsAg negative, Anti HBs positive, Anti HBc IgG positive) had 54% male and 46% female individuals with 54±13 of average age (Table 1).

Genotyping Analysis

Among the 19 analyzed DPB1 alleles in this study, DPB1*02:01 was the most prevalent (found in 30.2% of total patient and seroconverted group). On the other hand, HLA-DPB1*18:01, HLA-DPB1*02:02 and HLA-DPB1*09:01 were rare alleles in both groups. The frequencies of all alleles were given in Table 2. Representative image of agarose gel for RLFP is shown in Figure 1.

HLA-DPB1*15:01 allele was more frequent in the spontaneous seroconverted control group compared to chronic hepatitis B patients (15.3% vs. 1.1%, $\chi^2 = 12.5$, OR = 0.06, 95% CI = 0.08-0.046 $P < 0.001$). DPB1*02:01 and DPB1*10:01 were the other alleles that found more frequent in the control group (38.8% vs. 22.3% $P = 0.02$ and 16.5% vs. 5.3% $P = 0.02$, respectively) (Table 3). However these two alleles' associations lost their significance after Bonferoni's correction ($Pc = 0.4$ for all).

Discussion

Chronic Hepatitis B Infection is a global health problem, more than 2 billion people all around the world have been infected with the virus and more than 350 million people are chronically infected and are at in risk of developing liver cirrhosis and hepatocellular carcinoma (1). Studies have shown that HLA class II molecules, which show high polymorphism, are the susceptibility 6 factors of chronic HBV infection. Recent genetic association studies have shown that HBV infection and the progression of HBV related liver diseases are associated with the variations observed in the HLA-DP region of the genome especially HLA-DPA1 and HLA-DPB1 region (13, 14, 23). Candidate SNPs of the HLA loci that are related with chronic HBV infection, hepatic fibrosis and hepatocellular carcinoma were determined with previous GWA studies (27, 28).

Our study investigated the presence of possible variations in the HLA-DP region in Turkish population associated with HBsAg seroconversion. We analyzed two groups of subjects. First group consisted of a total of 94 patients with chronic HBV infection and the control group consisted of 85 HBsAg negative, anti-HBs positive and anti–HBc IgG positive spontaneous

seroconverted healthy subjects. In this line, our work aims to determine alterations in the HLA-DPB1 region of the genome of HBV infected Turkish patients and link these possible polymorphisms to disease outcome in Turkish population. In our study, frequencies of the 19 specific HLA-DPB1 allele polymorphisms were analyzed in patients with HBV infection using PCR-RFLP method. Among the 19 analyzed DPB1 alleles in this study, DPB1*15:01 allele was more frequent allele in the spontaneous seroconverted control group compared to chronic hepatitis B patients (15.3% vs. 1.1%, $\chi^2 = 12.5$, OR = 0.06, 95% CI = 0.08-0.046 $P < 0.001$, $Pc < 0.001$). DPB1*02:01 and DPB1*10:01 were the other alleles genotyped more frequently found in the control group (38.8% vs. 22.3% $P = 0.02$ and 16.5% vs. 5.3% $P = 0.02$, respectively). However, after Bonferroni correction, only DPB1*15:01 maintained its significance. This indicates an association of HLA DPB1*15:01 allele with spontaneous HBsAg seroconversion. It can be suggested that people carrying HLA-DPB1*15:01 allele may have a stronger immune response to HBV that leads to spontaneous seroconversion of HBsAg which is the ultimate target in chronic hepatitis B. This study revealed an association of HLA DPB1*15:01 allele with spontaneous HBsAg seroconversion. 7

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