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ARAŞTIRMA / RESEARCH

Effect of FOXP3 gene variants on the immune-active HBV and inactive HBV phases

FOXP3 gen varyantlarının immün-aktif HBV ve inaktif HBV fazları üzerindeki etkisi

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Abstract

Purpose: FOXP3 gene rs2232365 A/G and the rs3761548 A/C polymorphisms were associated with immune system-related diseases such as Hepatitis B virus (HBV) infection. The function of Treg cells which act as immune-suppressors in the control of HBV-related liver inflammation may be affected by these polymorphisms. The aim of the present study was to evaluate the association between these polymorphisms with HBV infection phases.

Materials and Methods: The current study examined the FOXP3 gene polymorphisms in 116 patients with immune-active hepatitis B phase and in 116 individuals with inactive hepatitis B phase by a real-time polymerase chain reaction (RT-PCR).

Results: In females, the A allele and AA genotype of rs2232365 polymorphism was not statistically significant although it increased 1.28- and 1.67-fold immune-active HBV risk. Although the G allele of rs2232365 polymorphism increased 1.69-fold immune active HBV risk, it was not statistically significant in males, either. Likewise, the rs3761548 polymorphism could not reach a statistically significant value in males and females, either.

Conclusion: This research is to demonstrate the relation between phases of HBV infection and polymorphisms of the FOXP3 gene in the Turkish population. The results of this study showed that there is no effect of these polymorphisms on the immune-active phase of HBV, even though it increased immune-active HBV.

Keywords: FOXP3, rs2232365, rs3761548, immune-active HBV, inactive HBV

Öz

Amaç: FOXP3 geni rs2232365 A/G ve rs3761548 A/C polimorfizmleri, Hepatit B virüsü (HBV) enfeksiyonu gibi bağışıklık sistemi ile ilgili hastalıklarla ilişkilendirilmiştir. HBV ile ilişkili karaciğer iltihabının kontrolünde immün baskılayıcı olarak görev yapan Treg hücrelerinin işlevi bu polimorfizmlerden etkilenebilir. Bu çalışmanın amacı, bu polimorfizmlerin HBV enfeksiyon evreleri ile ilişkisini değerlendirmektir.

Gereç ve Yöntem: Bu çalışmada, gerçek zamanlı polimeraz zincir reaksiyonu ile immün aktif hepatit B fazında olan 116 hasta ile inaktif hepatit B fazında olan 116 hastada FOXP3 gen polimorfizmleri incelenmiştir.

Bulgular: Kadınlarda, rs2232365 polimorfizminin A alleli ve AA genotipi, immün aktif HBV riskini 1.28 ve 1.67 kat artırmasına rağmen istatistiksel olarak anlamlı değildi. Erkeklerde ise rs2232365 polimorfizminin G alelinin immün aktif HBV riskini 1.69 kat arttırdığı ancak istatistiksel olarak anlamlı olmadığı bulunmuştur. Aynı şekilde rs3761548 polimorfizmi de erkek ve kadınlarda istatistiksel olarak anlamlı bir değere ulaşamamıştır.

Sonuç: Bu araştırma, Türk popülasyonunda HBV enfeksiyon evreleri ile FOXP3 gen polimorfizmleri arasındaki ilişkiyi gösteren bir çalışmadır. Mevcut çalışmanın sonuçları, bu polimorfizmlerin immün-aktif HBV riskini artırmasına rağmen immün-aktif HBV fazı üzerinde etkisinin olmadığını göstermiştir.

Anahtar kelimeler: FOXP3, rs2232365, rs3761548, immün-aktif HBV, inaktif HBV

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INTRODUCTION

The hepatitis B virus (HBV) affects a lot of people. Liver failure, liver cirrhosis and cancer can develop in approximately 15% of people infected with HBV infection. According to the current American Association of the Study of Liver Diseases (AASLD) guidelines, phases of chronic HBV (CHB) infection are divided into 3 categories; immune-tolerant phase, immune-active phase and inactive phase[1,2]. HBV phases are defined according to hepatitis B virus count, the e antigen of HBV (HBeAg) situation, and alanine amino transaminase levels[3]. The initial phase of CHB is called the immune-tolerant phase, which has normal liver histology and alanine aminotransferase (ALT) levels. Additionally, these patients have HBeAg positivity and high levels of HBV DNA.

The second phase is the immune-active or the immune-clearance phase that has HBeAg positive or negative, but seen liver inflammation, high ALT levels and lower HBV DNA levels compared with the immune-tolerant phase. As the final phase, the inactive HBV phase or immune control phase has HBeAg-negative, anti-HBeAg-positive, low HBV DNA levels and normal ALT levels[3,4].

Chronic HBV infection phases or HBV clearance are dependent on viral variations, the age and sex of the host, and genetic variants in the host-related immune system[5,6]. The increased count of circulating Treg cells accounts for the inadequate $CD8^+$ T cell response, and as a result, develops chronic HBV infection[7]. The forkhead box P3 (FOXP3) protein expressed by the *FOXP3* gene is responsible for the development and function of regulatory T (Treg) cells that regulate the immune system[8].

Treg cells have a vital role in immune homeostasis and provide immune-tolerance against self-and non-self of antigens[9]. $CD4^+CD25^+$ $FOXP3^+$ Treg cells inhibit other T cells by binding interleukin-2, a T cell growth factor, with CD25 molecule in the inflamed region[8,9]. Moreover, the CTLA4 molecules of Treg cells contribute to this process through binding the CD80 and CD86 molecules expressed on the surface of antigen-presenting cells[10]. Thereby, Treg cells prevent an aggressive T cell response to antigens and provide immune homeostasis. FOXP3 expression' down-regulation or dysfunction is related to immunological and inflammatory diseases such as multiple sclerosis[11], rheumatoid arthritis[12], vitiligo[13], ulcerative colitis[14], and chronic HBV infection[15].

The polymorphisms that existed in the promoter region of the gene may influence *FOXP3* gene expression by interaction with cis-acting elements and transcription factors (GATA-3, E47, c-Myb). Therefore, the Treg cell activation and function can abrogate[16,17]. This study explored the *FOXP3* gene rs2232365 and rs3761548 promoter polymorphisms in 116 patients with immune-active hepatitis B phase and 116 individuals with inactive hepatitis B phase in a Turkish population.

MATERIALS AND METHODS

This work was endorsed by the Ethics Committee of the University of Çukurova (number and date of ethical approval: 49 and 9 January 2016) in Turkey. Patients were included in the study at the Department of Gastroenterology, Balcalı Hospital, University of Çukurova from November 2016 until October 2020.The participants in this study signed an informed consent form respecting the usage of their blood samples. This study was realized according to the Helsinki declaration endorsed at the World Medical Association gathering in Edinburgh.

Both groups were selected according to current AASLD guidelines[18] HBeAg positivity or negativity, liver inflammation and damage, high alanine aminotransferase (ALT) levels, and low or high HBV DNA levels were detected in 116 patients with immune-active HBV phase. As a control group, 116 individuals in the chronic inactive hepatitis B phase had HBeAg-negative, anti-HBeAg-positive, low HBV DNA levels, and normal ALT levels. The patients who have co-infected with HIV, HCV, HDV, and patients with immunological diseases and the immune-tolerant HBV phase were excluded from this work.

The blood of the patients was taken at the time of diagnosis. All patients were treatment-naive patients and did not receive any medication that would affect the number and function of Treg-cells. The FOXP3 variants of all subjects were established by RT-PCR. Biochemical parameters and HBV DNA levels of all participants were established for analysis. The medical patterns of individuals were frozen at -80 ^oC until the experiment.

Isolation of genomic DNA, and the rs2232365 and rs3761548 variants' analysis

Genomic DNA from all study subjectswas extracted

from their blood samples according to the PCR kit's prospectus.

The alleles of these variants were identified by RT-PCR. RT-PCR protocol, in brief, 6 µl (about 100 ng) extracted genomic DNA was mixed by 13 µl of Roche Master, 4 µl of deionized water and 2 µl of primers-probes reagent in a total volume of 25 µl. Afterward, the RT-PCR process was implemented as the first denaturation at 95 °C (9 min), and whereupon throughout 43 cycles, denaturation at 95 °C (12 s), annealing at 58 °C (12 s), and extension at 72 °C (15 s), and ultimate melting conditions were at 95 °C (30 s) and 42 °C (4 min). The melting peaks of variants at a specific temperature as follows: the A allele of rs2232365 at 66.02 °C and the G allele of rs2232365 at 60.01 °C; the A allele of rs3761548 at 66.03 °C and the C allele of rs3761548 at 60.42 °C). To ensure quality control, the genotyping was done in the absence of disease situation information of the individuals, and 20% of random specimens of participants were determined twice by diverse experts, and reproducibility was 100 %.

Statistical analysis

The sufficient sample size and 80% statistic power were calculated with Quanto software using minor allele frequencies of these polymorphisms in HapMap, the prevalence of the disease in the population, and the effect of minor allele on phenotype. All analysis was got by using the IBM SPSS software (USA). Comparisons on demographical variables between both groups were done the Independent samples t-test or Mann-Whitney U test for continual variable, and chi-square test for categorical variables. Regression analysis with adjustment for age and gender was got to establish risk determinants in statistic models between the groups. Analyzes were two-sided. P values < 0.05 were accepted as statistically significant.

RESULTS

A total of 232 patients with chronic HBV were surveyed. While 116 of these patients were in the immune-active HBV phase, the other 116 were in the inactive HBV phase. The median value of HBV DNA levels was significantly higher in the immune-active HBV group than in the inactive HBV group (2.10^5 versus 413, P=0.014). The differences between the other variables of both groups were significant including age, haematocrit (HCT), leukocytes, albumin, gammaglutamyl aminotransferase (GGT), and aspartate aminotransferase (AST) levels (Table 1).

The total allele distributions of rs2232365 polymorphism were 49.3% and 50.7% for A and G, respectively. Furthermore, rs3761548 polymorphism total allele frequencies were 55.8% and 44.2% for C and A, respectively. A relationship was not established for the groups in the allele frequencies of these polymorphisms in females, males, and the study group (Table 2). As well, no significant differences were observed between the two groups in terms of allele frequencies and genotypes of these polymorphisms in females (Table 2).

Table 1. Distribution of selected characteristics in both groups.

Variable	Immune-active HBV group (%), n =116	Inactive HBV carriers group (%), n=116	*P* value
Age †	53.50 (21-89)	47.50 (18-76)	0.001*
Gender, male (%)	80 (69)	67 (57.8)	0.07**
Alcohol consumption , (%)	11 (6,2)	9 (5.9)	0.91**
AST(U/L) ‡	42.88 ± 8.23	28.70±6.25	0.001*
ALT (U/L) ‡	43.96 ± 11.23	35.57±8.10	0.18*
GGT (U/L) ‡	36.69±43.19	24.07±46.16	0.002*
Albumin (g/dL) ‡	3.92 ± 0.81	4.15 ± 0.45	0.01§
HCT(uL) ‡	40.10 ± 5.90	41.68 ± 5.38	0.03§
Leukocyte, ($x10^3$/ul) ‡	7.52±2.20	6.81±2.10	0.03*
HBV DNA, (IU/ml) †	2.10^5 (200-1.7x10^8)	413(20-1.63x10^3)	0.014*

*P values were estimated with the Mann-Whitney test. **P values were estimated with the chi-square test. §P values were estimated with the student t-test. †Median (min-max). ‡Mean±SD.

Table 2. The relationship among *FOXP3* polymorphisms and the immune-active HBV risk

***FOXP3* gene**	**Immune-active HBV, n =116, (%)**	**Inactive HBV carriers, n =116, (%)**	**P-value**	**OR (95% CI)**
For rs2232365,				
Total Allele frequency, n (%)				
A	70 (46.1)	79 (47.9)		1.00 (Reference)
G	82 (53.9)	86 (52.1)	0.75*	1.08 (0.69-1.67)
For rs3761548,				
Total Allele frequency, n (%)				
C	84 (55.3)	93 (56.4)		1.00 (Reference)
A	68 (44.7)	72 (43.6)	0.84*	1.05 (0.67-1.63)

*P values were estimated with logistic regression analysis. Adjustments were performed for age, gender, and alcohol consumption. Since this gene is on the X chromosome, males are represented by a single allele. Therefore, males are included with only 1 allele in the calculation of total allele frequency.

To explore whether an increased risk of immune active HBV by these variants, we implemented logistic regression analysis with adjustment for age and alcohol consumption between both groups in males and females (Table 3). In females, the A allele and AA genotype of rs2232365 polymorphism was not statistically significant although they increased 1.28- and 1.67-fold immune-active HBV risk, respectively (P=0.43 and P=0.45) (Table 3). Contrary, although the G allele of rs2232365 polymorphism increased 1.69-fold immune active HBV risk, it was not statistically significant in males (Table 3). Likewise, although the A risk allele of rs3761548 polymorphism increased the risk of immune active HBV, it could not reach a statistically significant value in males and females (Table 3).

Table 3. Analysis of *FOXP3* polymorphisms and immune-active HBV risk by the gender.

rs2232365	**Allele/Genotype**	**Immune-active HBV, n (%)**	**Inactive-HBV carriers, n (%)**	**P value**	**OR (95% CI)**
Male, n (%)		80 (69)	67 (57.8)		
	A	34 (42.5)	36 (53.7)	--	1.00 (Reference)
	G	46 (57.5)	31 (46.3)	0.14*	1.69 (0.84-3.37)
Female, n (%)		36 (31)	49 (42.2)		
	G	36 (50)	55 (56.1)	--	1.00 (Reference)
	A	36 (50)	43 (43.9)	0.43*	1.28 (0.69-2.36)
	GG	10 (27.8)	13 (26.5)	0.25*	1.00 (Reference)
	AG	16 (44.4)	29 (59.2)	0.51**	0.71 (0.25-2.00)
	AA	10 (27.8)	7 (14.3)	0.45**	1.67 (0.45-6.12)
Dominant	GG/AG+AA	10/26	13/36	0.83**	0.90 (0.33-2.41)
Recessive	GG+AG/AA	26/10	42/7	0.18**	2.09 (0.70-6.34)
Overdominant	AA+GG/AG	20/16	20/29	0.21**	0.57 (0.23-1.37)
rs3761548					
Male, n (%)		80 (69)	67 (57.8)		
	C	43 (53.8)	37 (55.2)	--	1.00 (Reference)
	A	37 (46.2)	30 (44.8)	0.63*	1.18 (0.60-2.36)
Female		36 (31)	49 (42.2)		
	C	41 (56.9)	56 (57.1)	--	1.00 (Reference)
	A	31 (43.1)	42 (42.9)	0.98*	1.01 (0.55-1.86)
	CC	14 (38.9)	17 (34.7)	0.71*	1.00 (Reference)
	CA	13 (36.1)	22 (44.9)	0.50**	0.71 (0.26-1.93)
	AA	9 (25)	10 (20.4)	0.83**	1.14 (0.35-3.68)
Dominant	CC/CA+AA	14/22	17/32	0.70**	0.84 (0.34-2.08)
Recessive	CC+CA/AA	27/9	39/10	0.56**	1.36 (0.48-3.90)
Overdominant	CC+AA/CA	23/13	27/22	0.39**	0.68 (0.59-2.03)

*P values were estimated with the chi-square test. **P values were estimated with the logistic regression. Adjustment was done by age and alcohol consumption.

Table 4. Frequencies of haplotypes of rs2232365 and rs3761548 variants in females.

Haplotypes *FOXP3* rs3761548 C/A and rs2232365 A/G	Frequency		OR (95%CI)	P-value
	Immune-active HBV	Inactive HBV		
CA	0.470	0.439	1.00 (Reference)	--
AG	0.400	0.429	0.88 (0.45-1.70)	0.70
CG	0.099	0.133	0.68 (0.21-2.22)	0.53
AA	0.030	0.000	18.81 (0.0-4893)	0.81

Global haplotype association P-value= 0.59

When we perform a risk analysis of total allele frequency regardless of gender, carrying the G risk allele of the rs2232365 polymorphism increases the risk of immune-active HBV by 1.08 times, but it is not statistically significant. Similarly, the A risk allele of the rs3761548 polymorphism increases the risk by 1.05 times, but was not considered significant either (Table 2). Additionally, in the haplotype analysis that we performed, no significant relationship was found (Table 4).

DISCUSSION

We investigated for the first time the effect of the *FOXP3* gene polymorphisms (rs2232365 and rs3761548) on the risk of immune active HBV phase predisposition using a case-control group in a Turkish population which is a Caucasian population. So far, only two studies have linked these polymorphisms with HBV infection. One of these studies is the article we previously investigated in terms of chronic HBV and spontaneous clearance[15], and another study explored the association between chronic active hepatitis B and chronic inactive hepatitis B groups[19]. Pereira et al. reported that the immune-active HBV patients with the rs2232365 GG genotype had lower viral loads and higher high liver enzyme levels compared to patients with the AA and AG genotypes when the immune-active HBV group compared within itself according to genotype.[19]. In addition to this, Pereira et al. determined high viral load along with low and medium levels of liver enzymes and absent/mild inflammation in the patients having the AA genotype. Moreover, in the same study, the chronic hepatitis C patients with the A allele of rs3761548 polymorphism had low viral loads together with moderate to severe inflammation[19]. Contrary, when the immune-active HBV group and the inactive HBV group were analyzed in terms of these polymorphisms, no significant difference was found[19]. In our previous study, although these polymorphisms increased the risk of developing chronic HBV infection, we found no statistically significant differences between these polymorphisms and those groups that are chronic HBV and spontaneous clearance.[15]. There is no study other than these studies investigating the relationship between HBV and these polymorphisms. Therefore, these polymorphisms of the *FOXP3* gene were selected as the aspirant polymorphisms because of their vital role in immune-related HBV disease.

In the current study, total allele distributions of these variants in the control group that is inactive HBV phase were got 47.9% and 56.4% for rs2232365 A and rs3761548 C variants, respectively. The allele distributions of rs2232365 and rs3761548 polymorphisms were not statistically significant between both groups, although they increased the risk of immune-active HBV. In the current study, the individuals having G allele of rs2232365 polymorphism had a 1.08-fold higher immune active HBV risk than persons who haven't got G allele, but not significant (P=0.75 OR=1.08 (0.69-1.67)). As for rs3761548, this polymorphism's the A allele increased a 1.05-fold immune-active HBV risk when compared to inactive HBV patients, but not significant (P=0.84). In the study reported from Brazil, these variants' total allele frequencies were not statistically significant between active HBV and inactive HBV groups, but they were significant in terms of histological activity and liver enzyme levels[19]. They found that both polymorphisms were significant for males when they compared these polymorphisms in groups of patients with hepatitis C and immune-active HBV by gender, but the sample numbers of the groups were insufficient for appropriate statistical analysis. Conversely, when we analyzed an association between these polymorphisms with immune-active HBV risk according to gender groups,

we didn't find any statistical significance for both polymorphisms albeit increased immune-active HBV risk (OR=1.69, P=0.14 for the G risk allele of rs2232365 in males, and OR=1.18, P=0.63 for the A risk allele of rs3761548 in males; OR=1.67, P=0.45 for the AA genotype of rs2232365 in females, and OR=1.14, P=0.83 for the AA genotype of rs3761548 in females). The risk allele for the rs2232365 polymorphism in males and females appears to be different.

This work also analyzed the association of these polymorphisms' genotypes with AST, ALT, GGT, leukocyte, and HBV DNA levels in the immune-active HBV group. However, no statistical significance was found for any of these blood values (data not shown). In contrast to us, Pereira et al. reported rs2232365 polymorphism to be associated with viral load and GGT levels, but not for AST and ALT enzymes. Another polymorphism rs3761548 was reported associated with low viral load and high GGT levels in hepatitis C patients[19].

It has been reported that FOXP3 gene expression is excessively increased in HBV patients with cirrhosis who have a very advanced immune-active HBV phase[20]. In addition to this, some studies reported that the FOXP3 expression was up-regulated in the inflamed colonic mucosa to prevent intestinal inflammation[14,21]. Especially, the rs2232365 and rs3761548 polymorphisms influence FOXP3 expression because of located within the DNA-binding sites of GATA-3 and Sp-1 transcription factors in the promoter region of the *FOXP3* gene[13,22]. Moreover, it was reported that the carrying risk alleles of rs2232365 and rs3761548 variants have lower FOXP3 expression, therefore lower FOXP3^{+}Treg cell frequencies in the patients with severe intestinal inflammation[14,23]. Thereby, in the case of low FOXP3 expression, regulatory T cells cannot inhibit excessive immune response, resulting in excessive inflammation and tissue damage.

A lot of studies investigated the relationship between these polymorphisms and diseases. The results of these works are controversial. Some studies declared the current polymorphisms significant concerning the disease they investigated, as others did not found an association. These diseases including breast cancer[24], crohn's disease[25], and psoriasis[26] were not significantly related to these polymorphisms, and these works are in parallel with the results of the present work. Contrast to us, others studies, renal diseases[27], ulcerative colitis,[14] and vitiligo[13], proclaimed an association with these polymorphisms. As males are represented by a single allele because of the X chromosome, perhaps that is why the results are different.

The current study has several limitations: a) all participants included in the current study were from Adana and surrounding provinces, whence, the present work does not represent all patients with HBV in the Caucasian population. b) Since the number of samples decreases in the relationship analysis with these polymorphisms in terms of gender, the statistical power decreases, so it is imperative to work with a larger sample number in the Caucasian population. c) This study only investigated the relationship of *FOXP3* polymorphisms with immune-active and inactive HBV phases. Therefore, the relationship of *FOXP3* polymorphisms with FOXP3 expression has not been established.

In conclusion, this research is the first to demonstrate the relationship between HBV infection phases and the *FOXP3* gene polymorphisms in the Turkish population. The results of this study demonstrated that these polymorphisms have no effect on the immune-active phase of HBV, even though the risk of immune-active HBV increases. However, it would be appropriate to conduct this research with a larger sample to confirm this result.

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