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Investigation of the Relationship Between *TNF-α*, *IL-12A*, *IL-12B* and *IFN-* γ Gene Polymorphisms and Chronic Hepatitis B in Turkish Populations

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Abstract:

Introduction: This study aimed to determine the relationship between TNF-α (-1031 T/C), IL-12A (3'UTR G/A), IL-12B (3'UTR A/C) and IFN-γ (+874 A/T) gene polymorphisms and chronic hepatitis B infections.

Materials and Methods: 100 patients with chronic hepatitis B and 100 healthy subjects were included in this study. Approximately 2 ml of peripheral blood from patient and control groups were taken into tubes with EDTA and the genomic DNA was isolated by using the DNA isolation kit. Single nucleotide polymorphisms (SNPs) in TNF-α (-1031 T/C), IL-12A (3'UTR G/A), IL-12B (3'UTR A/C) and IFN-γ (+874 A/T) genes were investigated by using RT-PCR method.

Results: It was determined that there was no statistically significant difference in terms of the allele and genotype frequencies of TNF-α (T/C) and IFN-γ (A/T) between chronic hepatitis B patients and the control group (p>0.05). However, there was a statistically significant difference between the patient and control groups for IL-12A (G/A) and IL-12B (A/C) in terms of genotype and allele distributions. The frequencies of IL-12A GG and GA genotypes (OR: 0.814, 95% 0.369-1.795, p=0.001) in patient group, and AA genotype in control group (OR: 2.608 95% 1.189-5.720, p=0.001) were high. Futhermore, it was found that the frequencies of IL-12A G allele in patient group and A allele in control group (OR: 1.981, 95%1.115-2.462, p=0.001) were high. In terms of IL-12B genotype and allele distributions, the frequencies of AC genotype (OR: 0.131, 95% 0.049-0.350, p=0.001) and A allele was higher in patient group than control group, whereas C allele in the control group was high. (OR:1.657, 1.115-2.462, p=0.012).

Conclusions: Our results showed that TNF-α (-1031 T/C), and IFN-γ (+874 A/T) polymorphism is not effective in chronic HBV infections. However, IL-12A (3'UTR G/A) and IL-12B (3'UTR A/C) polymorphisms may influence the chronicity of hepatitis B.

Key words: *Chronic hepatitis B, polymorphism, TNF-α, IL-12, IFN-γ, SNP*

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Introduction

Hepatitis B virus (HBV) infection is one of the most important causes of chronic liver. Chronic HBV infection (CHB) with HBV-related may cause cirrhosis, and hepatocellular carcinoma (HCC), and death (1). According to the Global Hepatitis Report (WHO 2017), it is estimated that the prevalence of HBV infection is 3.5%, and 257 million people have chronic HBV infection (2).

The host's immune response is important in the pathogenesis of HBV infections. There is an essential role of Th1 cells in protection against intracellular microorganisms such as bacteria and viruses (3). The most important cytokine in the differentiation of Th1 cells that fight intracellular pathogens is IFN-γ and IL-12. IFN-γ secreted from natural immune system cells activates T-bet and enhances Th1 differentiation by activating signal transducer and transcription 1 activator (STAT1). It enhances IFN-γ production by IL-12 STAT4-dependent pathway composed from active antigen-presenting cells and immune system cells. IL-12 also up-regulates its own receptor, increasing IFN-γ production through the IL-18 receptor and forming the cycle to increase Th1 effect. After Th1 cell differentiation, Th1 effector cells induce macrophage, natural killer cells, CD8+ T cells (IFN-γ, TNF-α). However, it is important that Th1 functions are in balance, if this balance is disrupted, inflammatory diseases and tissue damage may occur (3,4).

Polymorphisms in cytokine gene promoters can alter cytokine production. In many studies, it was showed that there is a relationship between cytokine gene polymorphisms and viral hepatitis. Furthermore, polymorphisms in the cytokine gene can influence serum level of cytokine and its expression (5-7).

Tumor necrosis factor-alpha (TNF-a) is one of the important cytokines in the immunopathogenesis of HBV infection (5). Polymorphisms in the promoter region of *TNF-α* gene may effect prognosis of the disease in patients with HBV infection (8-11). There are various polymorphisms in the promoter region of the *TNF-α* gene such as (-238G>A, -308G>A, -857C>T, -1031T>C (12). Many studies have shown that various polymorphisms in *TNF-α* affect both the persistence risk of the disease and the risk of cancer or cirrhosis (9,11,12). However, there are a few studies investigating the relationship between *TNF-α* -1031 promoter gene polymorphism and chronic hepatitis B in the literature (13,14). Additionally, there is no study that reveals the relationship between hepatitis B infection and *TNF-α* rs1799964 (-1031 T/C) polymorphism in the Turkish population.

Interleukin-12 (IL-12) which is one of the proinflammatory cytokines have two subunits (p40: heavy- chain) and (p35: short-chain). Two subunits of Interleukin-12 are encoded by IL-12A and IL-12B genes which are located on different chromosomes. IL-12 is mainly produced by macrophages, monocytes, neutrophils dendritic cells and B cells and their receptors are mainly located on the NK cells and the T cells. IL-12 stimulates Th1 response and increases IFN-gamma production and cell-mediated cytotoxicity (4,15). SNPs (*IL-12B* +1188A/C, *IL-12A* +277G/A, *IL-12A* -798T/A, *IL-12A* -504T/G, *IL-12A* -1148T/C) that may be associated with various diseases have been identified in the *IL-12A* and *IL-12B* genes and it is stated that these SNPs can affect the level of protein expression and cause various diseases such as autoimmune diseases, immune disorders, and cancer (16-18). However, there are very few studies investigating the relationship between these polymorphisms and hepatitis B infection in the literature (16,18,19).

Interferon-gamma (IFN-γ) plays an important role in defense against intracellular pathogens such as viruses and bacteria and in stimulating immune-related inflammatory responses. IFN-γ secreted by T cells and natural killer (NK) cells has many functions such as stimulating macrophage activation, mediating antiviral and antibacterial immunity, enhancing antigen presentation, organizing activation of the natural immune system, regulating Th1/Th2 balance and controlling cellular proliferation and apoptosis (20,21). The results of some studies showed that polymorphism of the IFN-γ+874 gene may be associated with chronic HBV infection and/or HCC. However, there is a difference in terms of allele and genotype distributions in a limited number of studies on this subject and this issue has not been understood enough (22-25).

Variations in cytokine genes differ in race, ethnicity and individual, and therefore allele and genotype distributions can also vary. There are limited studies on *TNF-α* (-1031 T/C), *IL-12A* (3'UTR G/A), *IL-12B* (3'UTR A/C) and *IFN-γ* (+874 A/T) polymorhisms in patients with hepatitis B infection in the literature (18,22,26). Therefore, this subject has not still understood. Besides, there is no study on this subject in our country. In this study, it was aimed to determine the relationship between *TNF-α* rs1799964 (-1031 T/C), *IL-12A* rs568408 (3'UTR G/A), *IL-12B* rs3212227 (3'UTR A/C) and *IFN-γ* rs2430561 (+874 A/T) gene polymorphisms and CHB infections in Turkish population.

Materials and Methods

Patient and Control Groups

A total of 200 subjects, 100 patients with CHB and 100 healthy subjects as control group were included in the study. All patients were recruited from outpatient of Infectious Diseases and Clinical Microbiology of Medicine Faculty in ….. University. Patients with HBV who were in accordance with HAI scores in histopathological examinations, were DNA (+), HBsAg (+), anti HBs (−), anti HBc IgG (+), HBeAg (±), and anti-HBe (±), whose ALT levels were one and a half to two times higher than their normal levels for at least 6 months. The control group was selected from healthy subjects who had HBsAg, anti-HBc, anti-HBs, anti-HCV and anti-HIV negative.

DNA Isolation and Genotyping

Approximately 2 ml of blood into a tube with EDTA was taken from the patient and control groups and genomic DNA was obtained using the DNA isolation kit (Roche High Pure DNA Isolation Kit, USA). Isolated DNAs were stored at 4°C until use. Real-time Polymerase Chain Reaction (qRT-PCR) method was used to determine single nucleotide polymorphisms (SNPs) in the *TNF-α* rs1799964 (-1031 T/C), *IL-12A* rs568408 (3'UTR G/A), IL-12B rs3212227 (3'UTR A/C) and *IFN-γ* rs2430561 (+874 A/T) gene region.

TNF-α, IL-12A and IL-12B Genotyping

In this study, qRT-PCR reaction mixture with a total volume of 20 µl was prepared. qRT-PCR reaction mixture contains 4 µl of sample DNA, 5 µl of ultra-pure water, 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems, ABD), 1 µl TaqMan assay. qRT-PCR reactions were carried out on a ViiA 7 qRT-PCR instrument (Applied Biosystems) following 1 cycles of 10 min at 95°C, 15 seconds at 95°C, and 40 cycles of 1 min at 60°C. Data were interpreted using Applied Biosystems Via 7 software.

IFN-γ Genotyping

The qRT-PCR reaction mixture for IFN-γ were prepared 10 µl LightCycler® 480 Probes Master Mix (Roche, USA), 4 µl PCR ultra-pure water, 5 µl DNA, 1 µl IFN-γ Light SNiP

assay (total volume:20 µl). The program of qRT-PCR was carried out on on the LightCycler® 480 qRT-PCR instrument by the following protocol: 95°C for 10 seconds for pre-denaturation, 45 cycles 95° C for 10 seconds, 57 °C for 20 seconds and 72°C for 10 seconds for amplification, and 95 °C for 30 seconds, 40°C for 2 minutes, 80°C for 0 seconds and 40°C for 30 seconds for cooling step, respectively. Data were interpreted using LightCycler 480 software.

Statistical analysis

Statistical analyses were done by SPSS statistical package. Chi-square and binary logistic regression tests were used in statistical analysis. Hardy Weinberg calculations were performed to control the balance of genotypes in groups and p values less than 0.05 were considered statistically significant.

Ethical issues

The study was approved by the clinical investigations ethics committee from Mersin University (Mersin University Clinical Research Ethics Committee, No: 2013/196) and informed consent was obtained from all the study participants.

Results

The mean age of the CHB patients was 46.41±14.39 (range:18-80), 42.0% were female and 58.0% were male. The mean age of the control group was 41.53 ± 12.87 (range:18-71), 46 46.0% were female and 39.4% were male. When the groups were compared in terms of age and gender, there was a significant difference in mean age, but gender distributions were homogeneous (p=0.012).

When the relationship between CHB and *TNF-α* rs1799964 (-1031 T/C) gene polymorphism was examined, TT, TC and CC genotypes were found in the patient group as 4.0%, 47% and 49% and in the control group as 6%, 34% and 60%, respectively (p=0.168) (Table 1).

The frequencies of T and C alleles were found to be 23% and 77% in the control group and 27.5% and 72.5% in the patient group, respectively (p=0.301) (Table 2).

The frequenciency of *IL-12A* rs568408 (3'UTR G/A) GG, GA, AA genotype was 22%, 49%, 29% in CHB patients and 16.0%, 29.0%, 55.0% in the control groups, respectively. There was a statistically significant difference between the patient and control groups for *IL12A* in terms of genotype distributions (p=0.001). According to this result, it was determined that the risk was reduced at 2.608 times in individuals with AA genotype. The frequency of G allele was 46.5% in the patients with CHB and 30.5% in the control groups. A allele frequency was 53.5% in the patient group and 69.5% in the control group (p= 0.001) (Table 1). While the frequency of A allele of *IL-12A* was higher in the control group, frequency G allele was higher in patient group. It was found that G allele increased the disease risk 1.981 times in CHB (p< 0.05) (Table 2).

The frequencies of AA, AC, CC genotype of IL-12B rs3212227 (3'UTR A/C) were found to be 6.0%, 94.0%, 0.0% in the patient with CHB and 20.0%, 41.0%, 39.0% in the control group, respectively. A statistically significant difference was found between the patient and the control group in terms of genotype distributions for IL-12B (p <0.001) (Table 1). In terms of IL-12B allele distributions, frequency of A allele was higher in patient group, C allele in control group (p=0.012). A statistically significant difference was found between the patient and the control

group in terms of allele distributions for IL-12B. It was determined that A allele increased the disease risk 1.657 times in CHB (p<0.001, p=0.012) (Table 2).

Table 1. Frequencies of *TNF-α* (-1031 T/C), *IL-12A* (3'UTR G/A), *IL-12B* (3'UTR A/C) and *IFN-γ* (+874 A/T) genotypes in chronic hepatitis B patients and control group.

Genotype	**Groups**				**P value**	**Confidence interval (95%)**
	Patients		**Control**			
TNF α (-1031 T/C)	**n**	**(%)**	**n**	**(%)**	0.168	
TT	4	4.0	6	6.0		**Reference**
TC	47	47.0	34	34.0		0.482 (0.126-1.842)
CC	49	49.0	60	60.0		0.816 (0.218-3.057)
Total	100	100	100	100		
IL-12A (3'UTR G/A)						
GG	22	22.0	16	16.0	0.001	**Reference**
GA	49	49.0	29	29.0		0.814(0.369-1.795)
AA	29	29.0	55	55.0		2.608(1.189-5.720)
Total	100	100	100	100		
IL-12B (3'UTR A/C)						
AA	6	6.0	20	20.0	<0.001	**Reference**
AC	94	94.0	41	41.0		0.131(0.049-0.350)
CC	0	0.0	39	39.0		*
IFN-γ (+874 A/T)						
TT	32	32.0	25	25.0	0.552	**Reference**
TA	39	39.0	45	45.0		1.477 (0.751-2.905)
AA	29	29.0	30	30.0		1.324 (0.638-2.750)
Total	100	100	100	100		

*: not determined

When the relationship between CHB and *IFN-γ* rs2430561 (+874 A/T) gene polymorphism was examined, TT, TA and AA genotypes were found in the patient group as 32.0%, 39.0% and 29.0% and in the control group as 25.0%, 45.0% and 30.0%, respectively (p=0.552) (Table 1). The frequencies of T and A alleles were found as 47.5% and 52.5% in the control group, and 51.5% and 48.5% in patient group, respectively (p=0.424) (Table 2).

Table 2. Frequencies of *TNF-α* (-1031 T/C), *IL-12A* (3'UTR G/A), *IL-12B* (3'UTR A/C) and *IFN-γ* (+874 A/T) allele in chronic hepatitis B patients and control group.

Allele	**Groups**				**P value**	**Confidence interval (95%)**
	Patients		**Control**			
TNF α (-1031 T/C)	**n**	**(%)**	**n**	**(%)**		
T	55	27.5	46	23.0	0.301	**Reference**
C	145	72.5	154	77.0		0.787 (0.501-1.238)
IL-12A (3'UTR G/A)						
G	93	46.5	61	30.5	**0.001**	**Reference**
A	107	53.5	139	69.5		1.981(1.315-2.983)
IL-12B (3'UTR A/C)						
A	106	53.0	81	40.5	**0.012**	**Reference**
C	94	47.0	119	59.5		1.657(1.115-2.462)
IFN-γ (+874 A/T)						
T	103	51.5	95	47.5	0.424	**Reference**
A	97	48.5	105	52.5		1.174(0.793-1.737)

In terms of gene-gene interaction; when *TNF-α*, *IL-12A*, *IL-12B* and *IFN-γ* genotypes in patients with chronic HBV patient group was compared to the control group, there was no significant difference (p>0.05) (Table 3).

Table 3. The interaction of *TNF-α* (-1031 T/C), *IL-12A* (3'UTR G/A), *IL-12B* (3'UTR A/C) and *IFN-γ* (+874 A/T) genotypes

TNF-α	**IL-12A**	**IL12B**	**IFN-γ**	**Control**	**Cases**	**OR**	**Lower 95 %CI**	**Upper 95 %CI**	**p**
TT	GG	AA	TT	6	4	Reference			
TC/CC	GG	AA	TT	10	2	0.300	0.042	2.165	0.232
TC/CC	GG	AC/CC	TT	0	16	2423212296.764	0.000	.	0.998
TC/CC	GA/AA	AA	TT	4	0	0.000	0.000	.	0.999
TC/CC	GA/AA	AC/CC	TT	5	10	3.000	0.571	15.766	0.194
TC/CC	GA/AA	AC/CC	TA/AA	75	68	1.360	0.368	5.025	0.645

Discussion

Cytokines play an important role in defence against HBV infections. It has been shown that polymorphisms in the cytokine gene affect the clinical course of the HBV (6,7,18). TNF-α production which affected by polymorphisms in the promoter region may influence the course of the disease in patients with HBV infections (11,14.15). Although there are many studies in the literature investigating the relationship between *TNF-α* gene (-238 G/A, -308-308 G/A, -863 C/A, etc) polymorphisms and HBV infections (7-9) but few studies have investigated the role of *TNF-α* rs1799964 (-1031 T/C) polymorphism in the immunopathogenesis of HBV infections (13,14). Besides, there was no study on polymorphism of these cytokine gene in patient with the chronic hepatititis B in our country. In our study, we investigated *TNF-α* rs1799964 (-1031 T/C) polymorphism in patients with CHB and there was no statistically significant

difference between the patient and the control group in terms of allele and genotype distributions. In a small number of studies investigating the relationship between *TNF-α* (-1031) polymorphism and the clinical course of hepatitis B disease (27,28), Jin et al. (13), investigated the *TNF-α* -1031 T>C, -857 C>T, and -308 G>A promoter polymorphism in HCC and liver cirrhosis patients. They found *TNF-α*-1031 T> C polymorphism was not associated with hepatitis B infection. In another study conducted by Liu et al. (26) was investigated *TNF-a* polymorphisms (-238G/A, -308G/A, -857C/T, -863C/A, -1031T/C) in CHB and HBV carrier individuals. It was found that the frequencies of GGCAT or GGTAT haplotypic in patients with CHB were significantly higher than in patients with self-limiting infection In another study by Tsuchiya et al. (14), allele frequencies (rs1799964 -1031 T/C and rs1800630 -863C/A) in the *TNF-α* promoter region in patients with fulminant hepatitis were found to be higher than in the control subjects ($p<0.05$). In contrast to our study, very few studies showed that polymorphism in the *TNF-α* rs1799964 (-1031 T/C) promoter region was significant in terms of allele and haplotype distributions in chronic HBV infections.

Several studies showed that there was an association between the *IL-2A* rs568408 (3'UTR G/A) and *IL-12B* rs3212227 (3'UTR A/C) polymorphisms and various diseases (16,29). However, there are a few studies investigating the association between these polymorphisms and CHB in the literature (18,19,27). But it has not yet been understood the relationship of IL-12 gene polymorphism with the persistence of hepatitis B virus infection. In our study, a statistically significant relationship was found between *IL-12A* rs568408 (3'UTR G/A) genotype and allele frequencies and CHB. The frequencies of *IL-12A* GA genotype and G allele were higher in patient group than control group. Furthermore, the frequency of *IL-12B* rs3212227 (3'UTR A/C) The frequency of AC genotype was higher in the patient group and CC genotype frequency was higher in the control group ($p<0.05$). A allele frequency was also higher in the patient group than control group ($p<0.05$). Tan et al (28) investigated the SNPs of the *IL-12A* (rs568406 and rs2243115) and *IL-12B* (rs3212227) in HBV-related HCC patients, persistent HBV carriers and subjects with HBV natural clearance from southern China. They showed that there is no a significant relationships between the rs2243115 and rs3212227 SNPs and HCC risk. Besides, they found that rs568408 variant genotypes were significantly associated with HBV-related HCC risk and susceptibility to HBV persistent infection. In a study by Liu et al. (18), it was investigated the relationship between *IL-12A* (rs568408, rs2243115), *IL-12B* (rs3212227) and HBV-related hepatocellular carcinoma in a Chinese population. They showed that the *IL-12A* rs568408 GA/AA variant genotypes were associated with a significantly increased risk of HCC. However, no significant relationship between *IL-12A* rs2243115 T/G, *IL12-B* rs3212227 A/C and risk of HCC were observed. In another study, Park et al. (27), investigated SNPs of *IL-12A* at

position +6400, +6624 and +7003 in Korean patients with HBV infection and healthy individuals who recovered from HBV infection. It was determined in their results that SNPs and haplotype of *IL-12A* were not associated with HBV persistence and development of HCC. Although the results of *IL-12A* and *IL-12B* gene polymorphisms differ in patients with hepatitis B in the literature, our study results showed that *IL-12A* rs568408 and *IL-12B* rs3212227 polymorphisms may be effective in the prognosis of hepatitis B disease. Genotype and allele differences may be due to various factors such as ethnic variations, viral disease agents, etc.

In our study, there was no statistically significant difference in *IFN-γ* rs2430561 (+874 T/A) genotypes and allele distributions between CHB patients and healthy subjects. Similarly, in a meta-analysis study by Wei et al. (22), it was implicated that there was relationship between the *IFN-γ* rs2430561 (+874T > A) polymorphism and the risk of HBV infection and that *IFN-γ* genetic polymorphisms might be important in determining an individual's susceptibility to HBV infection. In another study, Ghasemian and Shahbazi (30) investigated the relationship between the *IFN-γ* rs2430561 polymorphism and chronic HBV infection in the Iranian population and, they showed that there was no association between *IFN-γ*+874A/T polymorphism and chronic HBV infection. In a study by Naghizadeh et al. (23), it was determined that HBV infected patients with T allele have less risk to chronic HBV infection, whereas, A allele are more sensitive to chronic HBV infection. Furthermore, Al Kadi and Monem (31) investigated the association between *IFN-γ* +874 (T/A) polymorphism and chronic HBV infection in the Syrian population. Their results showed that the AA genotype increased the risk of chronicity, whereas the AT and TT genotypes reduced the risk of chronicity. A study conducted by Sun et al. (24) revealed that a significant association between *IFN-γ* polymorphisms (rs1861494, rs2430561) and HBV related liver cirrhosis (HBV-LC) risk in the Chinese population. In the results of the haplotype analysis, it was indicated that the T (+874) G (+2109) haplotype decreased the HBV-LC risk, and A (+874) A (+2109) haplotype increased the LC risk. In another study, it was found that a significant association between IFNγ +874 T allele and severe liver fibrosis when compared to mild fibrosis (6). Contrary to our study, although there are allele and genotype differences, it is seen that in most of the studies, there is a significant relationship between *IFN-γ* rs2430561 (+874 T>A) polymorphism and chronic HBV infection.

In our study, when the interaction of *TNF-α*, *IL-12A*, *IL-12B* and *IFN-γ* genotypes in patients with chronic HBV patient group was compared to the control group, there was no significant difference ($p>0.05$). Previously, it was determined that the interaction of allele with chronic HBV infection tremendously increased the risk of HCC (32). In another study by Gao et al.(33), interactions among polymorphisms of the genotype polymorphisms of *IFN–γ* +874 AA, *IL-2* -330 TT, *IL-10* -1082 AA, *IL-10* -592 AC and IL-4 -589 CC/CT were investigated and it

was determined that these genotypes are significantly influenced the clinical progression of hepatitis B. However, there is no further study on this subject in the literature.

Conclusions

According to our knowledge, this study is the first to determine the association between the *TNF-α* rs1799964 (-1031 T/C), *IL-12A* rs568408 (3'UTR G/A), *IL-12B* rs3212227 (3'UTR A/C) and *IFN-γ* rs2430561 (+874 A/T) gene polymorphisms in Turkish patients with CHB. Our results showed that *TNF-α* (-1031 T/C) and *IFN-γ* (+874 A/T) polymorphisms are not effective in chronic HBV infections, however, *IL-12A* rs568408 (3'UTR G/A), and *IL-12B* (3'UTR A/C) gene polymorphisms may influence the chronicity of hepatitis B. The difference in results might be attributed to the ethnic back ground of the studied populations, sample size, and the genotyping method. Therefore, more detailed studies with a larger sample size which are required to confirm the results in different regions may lead to an understanding of the molecular mechanisms of CHB infections.

Ethics Committee Approval: This study was conducted in accordance with the ethical standards
Informed Consent: NA
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