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TNF-alpha 308 SNP Rs3091256 GG Genotype is Strongly Associated with Fibrosis in Patients with Chronic Hepatitis C

TNF-alfa 308 SNP *Rs3091256* GG Genotipi Hepatit C Virüs Hastalarının Karaciğer Fibrozis Evreleri ile İlişkilidir

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ABSTRACT

Objective: We aimed to review the influence of host genetic factors on the clinical course, treatment response as well as fibrosis progression in patients with viral hepatitis C genotype 1.
Materials and Methods: Ninety-five patients with chronic hepatitis C virus (HCV) infection and 97 controls were enrolled. The patients received pegylated interferon (Peg-IFN)+ribavirin therapy for 48 weeks and were followed up for the next 48 weeks. Aspartat aminotransferase/platelet ratio (APRI) was used to detect liver fibrosis DNA specimens were extracted from the peripheral blood mononuclear cells and the tumor necrosis factor-alpha (TNF-α) 308 *rs3091256* was genotyped by the polymerase chain reaction-restriction fragment length polymorphism method.
Results: All patients included in the study were infected with HCV genotype 1. of the 95 HCV-positive patients, spontaneous viral clearence was observed in 25.5%, rapid viral response in 44.2%, early viral response in 91.8%, and sustained viral response was found in 73.3% of patients. The allele and genotype were not significant between patients and controls. There was no significant difference in virologic response as well. However, TNF-α-308 single nucleotide polymorphisms (SNP) *rs3091256* GG genotype was strongly associated with fibrosis and alanine aminotransferase (ALT) levels (p=0.006 and p=0.017, respectively).
Conclusion: TNF-α-308 polymorphisms may reveal different results among countries. Patients having SNP *rs3091256* GG are prone to have higher ALT levels and fibrosis score but have better treatment outcome.
Keywords: Hepatitis C, tumor necrosis factor alpha, polymorphisms, interferon, treatment

ÖZ

Amaç: Viral hepatit C genotip 1 hastalarında genetik faktörlerin klinik gidiş, tedavi cevabı ve fibrozis ilerlemesi üzerindeki etkisini gözden geçirmektir.
Gereç ve Yöntemler: Çalışmaya 95 kronik hepatit C virüs (HCV) hastası ve 97 sağlıklı gönüllü dahil edildi. Hastalar 48 hafta süreyle pegylated interferon (Peg-IFN)+ribavirin tedavisi kullandı ve sonraki 48 hafta boyunca takip edildi. Karaciğer fibrozis evresini belirlemek için aspartat aminotransferaz/platelet ratio (APRI) kullanıldı. DNA örnekleri periferik kan mononükleer hücrelerden izole edildi ve tümör nekroz faktörü-alfa (TNF-α) 308 rs3091256, polimeraz zincir reaksiyonu-kısıtlama fragmanı uzunluğu polimorfizmi yöntemi ile genotiplendi.
Bulgular: Tüm hastalar HCV genotip 1 ile enfekte idi. HCV hastalarının (95), %25,5 spontan viral klirensi, %44,2'si hızlı viral yanıt, %91,8'i erken viral yanıt ve %73,3'ünde kalıcı viral yanıt gözlendi. Hastalar ve kontroller arasında allel ve genotip açısından anlamlı fark yoktu. Virolojik yanıt da belirgin değildi. Bununla birlikte, TNF-α-308 tek nükleotit polimorfizmi (SNP) *rs3091256* GG genotipi fibroz ve alanin aminotransferaz (ALT) seviyeleri ile kuvvetli bir şekilde ilişkiliydi (sırasıyla p=0,006 ve p=0,017).
Sonuç: TNF-α-308 polimorfizmleri, ülkeler arasında farklı sonuçlar ortaya çıkarabilir. SNP *rs3091256* GG'ye sahip hastalar, daha yüksek ALT ve fibroz skoru göstermekle birlikte, bu popülasyonda daha iyi tedavi sonucuna sahiptir.
Anahtar Kelimeler: Hepatit C, tümör nekroz faktörü-alfa, polimorfizm, interferon, tedavi

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Introduction

Hepatitis C virus (HCV) is a major etiologic factor for the development of chronic liver diseases (cirrhosis, hepatocellular carcinoma, etc.). Hepatocytes are the primary target cells supporting HCV replication. After HCV infection, the innate immune system begins to respond to the virus and after 4 to 8 weeks, the CD8 + T cells recognize viral peptides that bind to human leukocyte antigen class I molecules in virus-infected hepatocytes (1).

This initiates signaling pathways leading to the synthesis of interferon (IFN), tumor necrosis factor (TNF) and a variety of other cytokines. In the acute phase of the infection, the virus is removed from the T-cell-mediated antiviral mechanisms. The rate of spontaneous viral clearance in acut HCV infection is aproximately 26% (range: 15-40%) (2,3,4,5). In patients who cannot clear the virus from hepatocytes in the first phase, HCV remains for years as long as it is not being treated. The effective treatment of chronic HCV infection (CHC) is based on a combination of pegylated-IFN (Peg-IFN) and ribavirin (RBV) (6). IFN, especially IFN- λ3, interacts with its acceptor, a heterodimer (IFN-lambdaR1 x IL-10R2). In IFN-based treatments, sustained viral response (SVR) rate is 40% (2,7).

The most important parameter in the therapeutic success in HCV infection is based on the HCV genotype (e.g., genotype 1 is the most difficult to treat) (2). In IFN-based treatments in the genotype 2, 3 and 5, the rate of SVR is 70-90% while in genotype 1 and 4, it is not more than 50% (1,8). HCV genotype 1 (91.8%) was the most common genotype in multicentre studie performed by Gürbüz et al. (9) in our country, while genotype 2 (4%) was detected in the second frequency.

Besides HCV genotype, serum alanine aminotransferase (ALT) level, histological grading and cytokine response of the host may affect HCV infection, viral clearance, and treatment (10,11). Among the cytokines, the most attention was devoted to TNF-alpha (TNF-α). Serum TNF-α level elevates in CHC patients (12) and SVR has been found to be associated with the baseline increased production of TNF-α (11). A positive correlation has been found between serum TNF-α levels and hepatic necroinflammatory score as well (13).

It was demonstrated that HCV can directly induce the expression of TNF-α in hepatocytes (14). Induction of TNF-α by HCV is dependent on Toll-like receptor (TLR) 7 and TLR8. Form recognition receptors seen in many cell types that participate in the innate immune response associated with viral infections and viral antigens are called TLRs (15). TNF binds to two receptors, TNFR1 and TNFR2; the first is structurally expressed in most cells, the second is inducible and has a more limited expression pattern (16). Upon receptor binding, TNF-α signals through a variety of cytosolic proteins, including TRADD (TNFR1-associated death domain protein) (17) and TNF receptor-associated factor 2 (18), leading to I_B degradation and the subsequent release and nuclear translocation of nuclear factor (NF)-κB. Binding of NF-κB to gene promoters initiates transcription of numerous proinflammatory cytokines, including TNF-α, IL-6, IL-8, and CXCL-10 (19,20) which suppress HCV replication.

The present study was designed to investigate the frequency of single-nucleotide polymorphism (SNP) of the TNF-308 locus in a population in Turkey, a region with a high prevalence of HCV infection. High prevalence of genotype 1b is more likely associated with fibrosing score, ALT level, spontaneous viral clearance and efficacy of treatment of HCV genotype 1.

Materials and Methods

A total of 95 anti-HCV-positive genotype 1b and 2 patients (70 HCV RNA+ chronic active hepatitis and 25 HCV RNA-negative and spontaneous clearance) and 97 healthy control subjects (57 female, 40 male) were included in the study. The Ethics Committee of Gaziosmanpaşa University approved the present study and all participants provided written informed consent for the study (Grant number: 11-BADK-111).

Genomic DNA was extracted from blood samples using an Invitrogen Genomic DNA Isolation Mini Kit K1820-02 (Invitrogen Life Technologies, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed in a total volume of 25 µL, using 100 ng genomic DNA with 20 pmol each primers, 0.2 mM each dNTP, 1X buffer, 2 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). Cycling was performed in a techne TC-4000 thermal cycler (Bibby Scientific Limited, Staffordshire, UK) as follows: amplification consisted of a 2-minute denaturation step at 96 °C; 35 cycles of one minute at 94 °C, one minute at 60 °C, one minute at 72 °C and final extension of 7 minutes at 72 °C followed by cooling to 4 °C.

Genotype analysis of TNF-α-308 (*rs3091256*) polymorphism was performed using restriction fragment length polymorphism. PCR products were digested with Nco1 restriction enzyme. The digested PCR products were resolved by electrophoresis on 2.5% agarose gels containing 0.5 µg/mL ethidium bromide. Restriction fragments were visualized with the use of a Vilber-Lourmat Gel Quantification and Documentation System QUANTUM-ST4 (Vilber Lourmat BP 66 Torcy, France). Aspartate aminotransferase/platelet ratio index (APRI) was used to determine liver fibrosis stage.

Statistical Analysis

Descriptive analyses were performed to provide information on general characteristics of the study population. Independent samples t-test was used to compare the continuous data between the groups. The continuous data were presented as mean ± standard deviation. Chi-square test was used to compare the categorical data between/among groups. Categorical variables were presented as a count and percentage. A p-value of less than 0.05 was considered statistically significant. Analyses were performed using SPSS 19 (IBM SPSS Statistics 19, SPSS Inc., IBM Co., Somers, NY).

Results

Ninety-five patients (62 female, 33 male) received Peg-IFN+RBV for 48 weeks and followed up for the next 48 weeks. APRI was used to determine liver fibrosis stage. There was no significant difference between the patient and the control groups in terms of age, gender and viral genotype. Clinical variables are given in Table 1. There was no statistically significant difference between CHC patients and healthy controls in terms of TNF-α-308 (*rs3091256*) polymorphism genotype distribution (p=0.362, Table 2). On the other hand, ALT values were found to be higher in CHC patients with TNF-α-308 GG polymorphism compared to CHC patients with GA+AA polymorphism (p=0.017). Moreover, APRI score was

Table 1. Clinical variables of the patients

Variables		Statistics
Patients	Active hepatitis	70 (73.7)
	Spontaneous clearence	25 (26.3)
RVY	hvy +	27 (42.2)
	hvy -	37 (57.8)
EVY	evy +	58 (90.6)
	evy -	6 (9.4)
Primary Non-responder	yes	6 (9.4)
	no	58 (90.6)
Relaps	yes	15 (23.4)
	no	49 (76.6)
SVR	yes	46 (71.9)
	no	18 (28.1)
Sex	female	63 (63.6)
	male	36 (36.4)
Treatment	IFN-α	35 (47.3)
	IFN-β	29 (39.2)
	No treatment	10 (13.5)
TNF	GG	78 (82.1)
	GA	14 (14.7)
	AA	3 (3.2)
TNF_Gene	GG	78 (82.1)
	GA+AA	17 (17.9)
IL28B860	CC	23 (23.2)
	CT	58 (58.6)
	TT	18 (18.2)
IL28B275	GG	18 (18.6)
	GA	53 (54.6)
	AA	26 (26.8)
IL28B917	GG	10 (10.1)
	GT	49 (49.5)
	TT	40 (40.4)
CCR5	NN	93 (93.9)
	ND	6 (6.1)
APRI score	0-0.49	43 (58.1)
	0.50+	31 (41.9)
APRI score	0.0	27 (36.5)
	No fibrosis	44 (59.5)
	Extensiv fibrosis	3 (4.1)
Age		55.91±9.84
HCV RNA		784.545±1345.413
HAI (ISHAK)		9.04±3.33
Fibrosing score (ISHAK)		1.83±1.05
WBC		6357.87±2192.49
HB		12.98±1.61
PLT		220.29±69.81
AST		38.7±28.07
ALT		44.85±42.02
APRI		0.56±0.42

Used to n (%) for qualitative variables and mean ± standard deviation for quantitative variables
RVY: Rapid virologycal response, EVY: Early virologic reponse, SVR: Sustained virologic response, APRI: Aspartate aminotransferase to platelet ratio index, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, TNF: Tumor necrosis factor, HAI: Hepatic activity index, WBC: White blood cells, HB: Hemoglobin, PLT: Platelets, HCV: Hepatitis C virus IFN-α: Interferon alpha, IFN-β: Interferon beta

found to be significantly higher in CHC patients with TNF-α-308 GG polymorphism compared to those with GA+AA polymorphism (p=0.006) (Table 3).

To evaluate the clinical applicability of the outlined SNP, we calculated the predictive odds ratios for the SNP between rapid virological response (RVR), early virological response (EVR) and SVR (Table 4). There were 24 patients who had spontaneous viral clearance. RVR was seen in 27 patients. EVR in 56 patients and SVR was observed in 44 patients. All the genotypes or alleles predicted the positive response to treatment in the overall study population.

Discussion

HCV infection continues to be a major health problem worldwide. Viruses are the most common cause of diseases such as chronic hepatitis and liver cirrhosis. HCV is divided into seven major genotypes (21). After infection with HCV, a large proportion of patients develop CHC, with a very few spontaneous clearance. (6). There are viral and host factors that are important in the development of chronic infection. Baseline viral load, RVR and host characteristics (e.g. alcohol consumption, steatosis, liver fibrosis, metabolic syndrome, ethnicity, and host genetic polymorphisms, especially IL28B, are the examples that have impact on virological response of the host (2). The most common HCV genotype worldwide is genotype 1 (46% of all HCV cases), while genotype 3 is the second most common (30%). However, the distribution of these genotypes varies between countries (22).

The first target for HCV is human hepatocytes. The immune system first activates the natural immune system. As a result, local IFN production begins and HCV genome replication and spreading in the liver parenchyma is disrupted (23). The mechanism of effective clearance of HCV from the human body is likely related to both environmental and host genetic factors. For example, it has been observed that treatment success in patients of European ancestry is better than in patients of African ancestry (24). In the current study, we performed an analysis to examine the association between the SNP in the promoter region of TNF-α-308 *rs3091256* and fibrosis score, ALT level, spontaneous viral clearance and treatment response to HCV infection.

There are varying results from studies on TNF-α-308 gene polymorphism in the literature. Although some studies have reported a significant association between TNF polymorphism and response to hepatitis C treatment, some studies reported

the opposite. In their study, Dai et al. (24) from Taiwan suggested that TNF polymorphism at position-308 may be a predictor of treatment failure in patients treated with a combination of IFN-α and RBV and in another study from Brazil, the TNF-α-308 a allele was found to be a predictor of null virological response (25,26), but other investigations have failed to confirm such findings. An Egyptian study found that at the TNF-α 308 position, the G/G allele was most common (78.5%) in the study population compared to controls (27).

In this study, no significant difference was observed in the frequency of the TNFα-308 (*rs3091256*) polymorphism. Our results are consistent with those reported by Barrett et al. (28) who did not find the SNP at 308 to be associated with viral recovery or persistence. In studies conducted in different countries, there was

Table 2. Summary of genotyping between patients completed antiviral treatment for chronic hepatitis C virus and control

Genotype/allele	Patients (95)	p for HWE	Control (97)	p for HWE	p	OR
GG	78 (0.8210)		82 (0.8454)		0.701	0.84 (0.39-1.79)
GA	14 (0.1474)		15 (0.1546)		1.000	0.94 (0.43-2.07)
AA	3 (0.0316)	**0.0339**	0	0.492	0.119	7.38 (0.38-142.61)
G	170 (0.8947)		179 (0.9227)		0.378	0.71 (0.35-1.43)
A	20 (0.1053)		15 (0.0773)			

OR: Odds ratio, HWE: Hardy-Weinberg equilibrium

Table 3. Comparison of qualitative and quantitative variables between to GG and GA+A

Variables		TNF		p
		GA+AA	GG	
Non-responder	Yes	4 (7.8)	2 (18.2)	0.287[a]
	No	47 (92.2)	9 (81.8)	
Relapse	Yes	12 (23.5)	2 (18.2)	0.525[a]
	No	39 (76.5)	9 (81.8)	
SVR	Yes	37 (72.5)	8 (72.7)	0.652[a]
	No	14 (27.5)	3 (27.3)	
APRI		0.61±0.46 (n=58)	0.39±0.17 (n=13)	**0.006**[b]
ALT		47.90±45.53 (n=78)	31.29±18.27 (n=17)	**0.017**[b]

Used to n (%) for qualitative variables and mean ± standard deviation for quantitative variables
[a]: Fisher's exact test, [b]: Independent samples t-test
SVR: Sustained virologic response, APRI: Aspartate aminotransferase to platelet ratio index, ALT: Alanine aminotransferase, TNF: Tumor necrosis factor

Table 4. Genotype and allele frequencieas for tumor necrosis factor-alpha 308 *rs3091256*

		Genotypes			Alleles	
		AA	G	A	GG	GA
Patients	CHC (n=70)	58 (82.86%)	10 (14.29%)	2 (2.86%)	126 (90.00%)	14 (10.00%)
p		1.000	1.000	1.000	1.000	
OR, 95% CI		0.97, 0.29-3.27	1.17, 0.30-4.54	0.68, 0.06-7.50	1.05, 0.36-3.05	
RVR	Yes (27)	25 (92.59%)	2 (7.41%)	0	52 (96.30%)	2 (3.70%)
	No (34)	26 (76.47%)	7 (20.59%)	1 (2.94%)	59 (86.76%)	9 (13.24%)
p		0.162	0.276	1.000	0.110	
OR, 95% CI		3.85, 0.77-19.33	0.31, 0.06-1.58	0.41, 0.02-9.81	3.97, 0.83-18.93	
EVR	Yes (56)	47 (83.93%)	8 (14.29%)	1 (1.79%)	102 (91.07%)	10 (8.93%)
	No (5)	4 (80.00%)	1 (20.00%)	0	9 (90.00%)	1 (10.00%)
p		1.000	0.563	1.000	1.000	
OR, 95% CI		1.31, 0.16-10.49	0.67, 0.08-5.42	0.30, 0.01-6.56	1.13, 0.14-8.93	
SVR	Yes (44)	36 (81.82%)	7 (15.91%)	1 (2.27%)	79 (89.77%)	9 (10.23%)
	No (16)	14 (87.50%)	2 (12.50%)	0	30 (93.75%)	2 (6.25%)
p		0.715	1.000	1.000	0.725	
OR, 95% CI		0.64, 0.13-3.26	1.32, 0.26-6.84	1.14, 0.05-27.06	0.59, 0.12-2.80	

RVR: Rapid virologycal response, EVR: Early virologic reponse, SVR: Sustained virologic response, CHC: Chronic hepatitis C, OR: Odds ratio, CI: Confidence interval

no correlation between *TNF* gene polymorphisms and histological severity or response to antiviral treatment (29). In a meta-analysis of studies performed at different centers, it has been shown that there is no significant association between TNF-α-308, -238 gene polymorphisms and susceptibility to infection among different HCV subgroups. (30). Besides, the distributions of TNF-α-308, -238 A/G alleles were also not significantly different between the persistent infection group and the spontaneous clearance group. It is well known that certain diseases such as psoriasis and concomitant HCV infection are succesfully treated with anti-TNF therapy without signs of reactivation of HCV (31,32). Therefore, we conclude that TNF-α-308 (*rs3091256*) polymorphism may not really have any effect on treatment response.

TNF-α may affect hepatic fibrogenesis by stimulating hepatic stellate cells (33). After TNF-α activation, Kupffer cells secrete TGF-beta1, an important fibrogenic molecule. The relationship between cirrhosis development and TNF promoter has been investigated extensively (34,35,36,37). Although Romero-Gómez et al. (38) found no association between polymorphism in -308 and the severity of fibrosis in HCV and Abdel-Latif found (11) in both fibrotic and cirrhotic cases, no significant correlation was observed in levels of matrix metalloproteinases (MMP)-2, MMP-9, and TNF-α between fibrotic and cirrhotic cases (39). TNF-α has been found higher in cirrhotic patients compared to CHC patients with no or mild fibrosis (40). Consistent with these results, our study also confirmed the association between TNF-α-308 GG polymorphism and fibrosis score (p=0.006). This may be explained by higher constitutive and inducible transcriptional activity of TNF. Nevertheless, in a meta-analysis of 11 different studies, no association was found between TNF-α-308G> A polymorphism and liver cirrhosis risk in both Caucasians and Asian populations (41).

No correlation has been shown between 308 promoter polymorphisms and necroinflammatory histological activity. Although one study compared ALT levels between SVR patients and non-responders and found no statistically significant difference (10), the other found a statistically significant difference between healthy controls and those with cirrhosis and hepatocellular carcinoma (30), these studies did not include TNF polymorphism. Abbas et al. (41) studied HCV genotype 3 and found no association between ALT level and TNF-α-308 polymorphism. Besides, only 5% of their patients had TNF-α-308 GG promoter. In our study, we found a statistically significant relationship between TNF-a-308 GG polymorphism and high ALT levels in HCV genotype 1 patients (p=0.017) and although not statistically significant, 73.3% of our study population had SVR. In a study from Turkey, liver infiltrating lymphomononuclear cells were stimulated with TNF-α and histology activity index and HCV genotype revealed a negative correlation between TNF-α levels and elevated ALT levels in patients infected with 1b (42).

After circulating HCV particles reach the basolateral surfaces of hepatocytes, where the virus first binds to several receptors, the virus attaches to hepatocytes, it fuses the membrane and enters the cytosol and starts to replicate (43). Liver damage from HCV depends on both host's immune system-mediated reactions and viral cytopathic effects (44). The CD95 frequency was significantly higher in HCV antigen-positive hepatocytes compared to uninfected cells (45). TNF might play a role in hepatic necrosis and inflammation. Serum ALT level correlates with liver damage and we here propose that elevated ALT levels is the hallmark of hepatocyte injury eventully leading to fibrosis as well as elimination of virus in CHC (the more inflammation, the more viral eradication) (46).

Study Limitations

The study was conducted before the start of the use of new treatments.

Conclusion

In conclusion, this is the first article in which ALT level and liver fibrosis are associated with TNF-α-308 GG polymorphism treated with IFN. The discrepancies in TNF genetic polymorphism and treatment responses among studies may be due to differences between ethnic groups.

Ethics

Ethics Committee Approval: The Ethics Committee of Gaziosmanpaşa University approved the present study (approval number: 11-BADK-111).

Informed Consent: Informed consent forms were obtained from all the patients who participated in the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practise: Ö.G., A.R., Ö.A., Ş.B., Concept: Ö.G., D.Y., B.Ç., Desing: Ö.G., D.Y., B.Ç., Data Collection or Processing: Ö.G., A.R., Analysis: O.D., S.K., Literature Search: Ö.G., D.Y., B.Ç., Ö.T., Writing: Ö.G., D.Y., B.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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