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Effect of tumour necrosis factor-alpha and interleukin-6 promoter polymorphisms on course of Crimean-Congo hemorrhagic fever in Turkish patients

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ABSTRACT

Objective: In this case-control study, we investigated whether IL-6 (-174G/C) and TNF-α (-308G/A) gene polymorphisms affect the clinical course and outcome of CCHF.

Methods: Total 150 patients with CCHF and 170 controls were examined in this study. Genotyping of these polymorphisms were performed by PCR-RFLP methods.

Results: We found no statistically significant differences in genotype and allele frequencies of these polymorphisms between patients and controls [($\chi^2 = 1.31$, $p = 0.51$ for TNF-α) and ($\chi^2 = 2.61$, $p = 0.27$ for IL-6)]. Either TNF-α AA or IL-6 CC genotypes in dead cases were not observed in this study. Frequency of heterozygous genotypes in both IL-6 (GC) and TNF-α (GA) was higher in dead patients than living patients. However, the difference was not statistically significant. A significant difference was found in AST levels and INR when compared to patients with CCHF who died and who survived [OR = 13.9 (95% CI = 1.79–107) for INR, $p = 0.01$] and [OR = 23.3 (95% CI = 3.62–149) for AST, $p = 0.001$], respectively.

Conclusion: We did not find a significant association of IL-6 -174G/C and TNF-α -308G/A polymorphisms on the prognosis of CCHF and mortality in this study. We suggest that AST and INR may be important biomarkers for determining the risk of severity and death as a result of infection with Crimean-Congo hemorrhagic fever virus (CCHFV).

Key words Crimean-Congo hemorrhagic fever (CCHF); IL-6; polymorphism; TNF-α

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is a zoonotic viral disease. CCHF was first described in 1940s in farmers and soldiers in the Crimean peninsula of Soviet Union[1]. Turkey has been known to be endemic for human CCHF. In Turkey, patients infected with CCHFV were first reported in 2002. Since this year, CCHF outbreaks have been seen in endemic seasons and rural areas of Turkey[1–2]. Until now, the clinical course of the disease that may change from asymptomatic to fatal presentation, and pathogenesis of CCHF have not been clearly described[3]. Primary pathophysiological events of CCHF are erythrocyte and plasma leakage in the tissues due to damage of endothelial cells, coagulation dysfunction and bleeding. Various studies have suggested that indirect effect of high levels of some cytokines might cause these symptoms[4]. Single nucleotide polymorphisms (SNPs) in promoter region of cytokine genes have been shown to alter the level of cytokines and/or their activity[5].

The tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) genes are located within the class III region of the major histocompatibility complex on human chromosome 6p21 and 7p15, respectively. Several SNPs which influence the binding of transcription factors to promoters of these genes, may affect the host immune responses, mortality rate and clinical course of disease in various diseases[6–7]. Therefore, in the current study, we aimed to investigate whether promoter polymorphisms of TNF-α and IL-6 genes have an effect on the clinical course and outcome of CCHF.

MATERIAL & METHODS

Study group

One hundred and fifty patients with CCHF who reported at Department of Infectious Disease, Cumhuriyet University during 2007 to 2010, and 170 unrelated healthy controls were included in the current study. A written informed consent was obtained from both groups. Study was approved by the local ethics committee (numbers of the ethic committee decisions; 2010/023, 2011/023 and

2011/026). Case identification was made according to the criteria, established by the Turkish Republic Ministry of Health and CCHF working group. CCHFV was detected using polymerase chain reaction (PCR) method. All individual PCR results were positive for CCHFV in their serum samples which were selected as patient group. Severity scores for patients were carried out as described by Swanepoel *et al*[8] and Ribeiro *et al*[9]. Patients who have leukocyte count 10,000 mm^3 or above, platelet count ≤ 20,000/mm^3, level of aspartate aminotransferase (AST) ≥ 200 IU/l, alanine aminotransferase (ALT) ≥ 150 IU/l, activated partial thromplastin time (aPTT) ≥ 60 sec, international normalized ratio (INR) ≥ 1.4 sec and fibrinogen levels ≤ 110 mg/dl were accepted as severe cases. Controls were selected from healthy voluntary individuals who were not known to have any chronic disease.

Genotyping

Total genomic DNA was extracted from 200 µl of whole peripheral blood by using genomic DNA isolation kit (Macherey-Nagel, Germany) according to manufacturer's instructions. PCR and restriction fragment length polymorphism (RFLP) methods were used for detection of promoter polymorphisms of IL-6 gene and TNF-α gene. PCR amplification was performed on the Applied Biosystems Gene AmpR PCR system 9700 (USA) thermal cycler. PCR amplifications were made using a set of forward primer and reverse primer. The sequences of primer sets and PCR conditions are shown in Table 1. PCR was performed in a reaction volume of 25 µl containing 50 ng of genomic DNA, 10 pmol of the amplification primers for IL-6 (-174G/C) and TNF-α (–308 G/A), 5 nmol each of four deoxyribonucleotide triphosphates (Fermentas), 1 unit of Taq DNA polymerase (Fermentas), 10 mmol/l Tris-HCl (pH 8.3 at 25°C), 50 mmol/l KCl, and 1.5 mmol/l $MgCl_2$. The amplified products of 107 bp for TNF-α and 299 bp for IL-6 were digested with NcoI and NlaIII, respectively, according to the manufacturer's instructions (Fermentas) and then subjected to electrophoresis in a 3% agarose gel and visualized under UV light using ethidium bromide staining. The substitutions occurred in promoters of the IL-6 gene and TNF-α gene, generated three genotypes and two phenotypes as shown in Table 1.

Statistical analyses

Statistical analyses were performed using SPSS 15.0 programme. Independent-samples *t*-test was used to compare mean age among the groups. Genotype frequencies between patients and controls and among patient groups were analysed using χ^2-test. As estimation of relative risk of the disease, odds ratios (OR) were calculated on the basis of 95% confidence intervals (CI). *P*-values <0.05 were considered to be statistically significant. Analyses for deviations from Hardy-Weinberg equilibrium were performed based on the χ^2-test.

RESULTS

Demographic findings of the patient and control groups have been shown in Table 2. There was no significant difference between mean age and gender distribution of the patients and controls. However, mean ages of the dead patients were found to be greater than controls and survived patients (living). Mean laboratory findings of patients are shown in Table 2. Statistically sig-

Table 1. Primer set of amplifications for TNF-α (-308G/A) and IL-6 (-174G/C) polymorphisms, PCR conditions, RE and fragment sizes of PCR and RFLP products

Polymorphisms	IL-6 (-174G/C)	TNF-α (-308G/A)
Primer sequences	5'-TCCTCCCTGCTCCGATTCCG-3' 5'-AGGCAATAGGTTTTGAGGGCCAT-3'	5'-AGGCAATAGGTTTTGAGGGCCAT-3' 5'-TTGTCAAGACATGCCAAGTGCT-3'
PCR conditions	95°C–5 min ID	95°C–5 min ID
	95°C–30 sec 58°C–30 sec 35 cycles 72°C–45 sec…….	95°C–45 sec 59°C–45 sec 35 cycles 72°C–45 sec…….
	72°C–5 min FE	72°C–5 min FE
PCR product	299 bp	107 bp
RE enzyme	NlaIII (Fermentas)	NcoI (Fermentas)
RFLP products		
HWT	227, 59, 13 bp	87, 20 bp
HT	227, 118, 109, 59, 13 bp	107, 87, 20 bp
HPT	118, 109, 59, 13 bp	107 bp

ID–Initial denaturation; FE–Final extension; HWT–Homozygous wild type; HT–Heterozygous type; HPT–Homozygous polymorphic type.

Table 2. Demographic features and mean laboratory findings of all groups

Demographic features	Severe cases (n=67)	Mild cases (n=83)	Dead cases (n=19)	Living cases (n=131)	Patients (n=150)	Controls (n=170)
Mean age±SD	47.49±19 (18–80)	45.27±18 (17–82)	54±18 (18–80)	45.14±18 (17–82)	46.26±18 (17–82)	43.09±10 (18–70)
P-values	0.47		0.05		0.06	
Gender, n (%)						
Male	31 (46.3)	34 (41)	10 (52.6)	55 (42)	65 (43.3)	90 (52.9)
Female	36 (53.7)	49 (59)	9 (47.4)	76 (58)	85 (56.7)	80 (47.1)
P-values	0.61		0.46		0.09	
Laboratory findings	Severe cases (n=67)	Mild cases (n=83)	Fatal cases (n=19)	Non-fatal cases (n=131)	Patients (n=150)	References
Leucocyte count ($\times 10^9$/l)	4.2(0.7–27)	2.7(1–8)	5.1(0.7–22)	3.1(0.7–27)	3.4(0.7–27)	4–11
Platelet count ($\times 10^9$/l)	4(8–23)	11(30–187)	36.1 (10–86)	86.7(8–231)	80.3(8–231)	150–450
aPTT (sec)	24.2(9.7–151)	13.9(9.4–42)	45.4(10–151)	14.6(9.4–151)	18.5(9.4–151)	25.1–34.7
INR (sec)	1(0.8–2.4)	1 (0.7–1.3)	1.2(0.8–2.4)	1(0.7–1.6)	1(0.7–2.4)	
AST (U/L)	551(23–2439)	80(14–197)	585(82–1847)	247(14–2439)	290(14–2439)	9–36
ALT (U/L)	184(13–1076)	40(12–119)	188(67–661)	92(12–1076)	104(12–1076)	10–28
Fibrinogen (μg/dl)	224(46–326)	269(138–393)	206(89–299)	314(46–393)	340(46–393)	

nificant differences were observed at laboratory and bleeding findings in patient groups ($p<0.05$). The genotype distributions and allele frequencies of these genes and OR values among patients-controls and patients groups are summarized in Tables 3 and 4, respectively. Genotype frequencies of both the IL-6 and the TNF-α polymorphisms were not deviated from Hardy-Weinberg equilibrium ($p>0.05$). We found no statistically significant difference in genotype and allele frequencies of these polymorphisms between patients and controls [(χ^2=1.31, p=0.51 for TNF-α), (χ^2=2.61, p=0.27 for IL-6)]. Either TNF-α AA or IL-6 CC genotypes in dead cases were not observed in this study. Frequency of heterozygous genotypes in both IL-6 (GC) and TNF-α (GA) was higher in dead patients than living patients. However, the difference was not statistically significant. In addition, frequency of phenotype of high producing TNF-α (GA+AA) was found to be higher in dead patients (31.6%) compared to living patients (16.8%) but the findings were not statistically significant (p = 0.12). The comparisons of risk factors for severity of CCHF prognosis are shown in Table 5. A significant difference was found in AST levels and INR when compared to patients with CCHF who died and living [OR=13.9 (95% CI=1.79–107) for INR, p = 0.01] and [OR=23.3 (95% CI=3.62–149) for AST, p = 0.001], respectively.

DISCUSSION

We analysed the possibile relationship between promoter polymorphisms in IL-6 and TNF-α genes with CCHF severity and outcome in Sivas (Central Anatolia). Analysis of the data obtained in the current study showed that there was no statistically significant relationship among these two gene polymorphisms and development of disease and CCHF severity and mortality. We suggest that these polymorphisms have no direct effect on severity or development of the CCHF. In some studies supporting our finding, it has been reported that the IL-6 -174G/C polymorphism has no significant effect on the outcome or severity of several viral infections including chronic hepatitis B virus (HBV) infection and respiratory syncytial virus (RSV), bronchiolitis and HIV/AIDS[9–11]. In contrast, Fabris *et al*[12] for HBV infection, Cussigh *et al*[13] for hepatitis C virus (HCV) infection and Doyle *et al*[14] for experimental rhinovirus (RV39) infection, found a significant association between IL-6 promoter polymorphism and mentioned diseases. Barrett *et al*[15] have found that IL-6-174 polymorphism was associated with development of persistent HCV infection, however, TNF-α-308 polymorphism was not associated with outcome of the disease in this study. As, consistent with our finding, Loke *et al*[16] reported that there was no association between TNF-α-308G/A polymorphism and the risk of developing dengue hemorrhagic fever (DHF) among Vietnamese. Vejbaesya *et al*[17] investigated TNF-α and lymphotoxin-α (LTA) haplotype profiles and found no association between TNF-α-308A allele and severe dengue virus infection in Thais patients. In contrary to our data, Fernandez-Mestre *et al*[18] observed that the frequency of TNF-α-308A allele was increased > 5 times in Venezuelans with DHF. The homozygous geno-

Table 3. Distribution frequencies of IL-6 (-174G/C) and TNF-α (-308G/A) genotypes between patient and control groups and risk analysis results

Gene	Polymorphism	Genotypes	Phenotypes	Control n (%)	Patients n (%)	*p*-value	OR (95% CI)
IL-6	(-174G/C)	GG	High	111 (65.3)	85 (56.7)		Reference
		GC	High	57 (33.5)	62 (41.3)	0.16	1.42 (0.89–2.24)
		CC	Low	2 (1.2)	3 (2)	0.65	1.95 (0.32–11.98)
		G		279 (82.1)	232 (77.3)		Reference
		C		61 (17.9)	68 22.7)	0.14	1.34 (0.91–1.97)
TNF-α	(-308G/A)	GG	Low	139 (81.8)	122 (81.3)		Reference
		GA	High	30 (17.6)	25 (16.7)	0.88	0.94 (0.53–1.70)
		AA	High	1 (0.6)	3 (2)	0.34	3.41 (0.35–33.2)
		GA+AA		31 (18.2)	28 (18.7)	1	1.02 (0.58–1.81)
		G		308 (90.6)	269 (89.7)		Reference
		A		32 (9.4)	31 (10.3)	0.79	1.10 (0.65–1.86)

Table 4. Distribution frequencies of IL-6 (-174G/C) and TNF-α (-308G/A) genotypes among patient groups and risk analysis results

Patient groups	Living n (%)	Dead n (%)	*p*-value	OR (95% CI)	Severe n (%)	Mild n (%)	*p*-value	OR (95% CI)
IL-6 genotypes								
GG	78 (59.5)	7 (36.8)		Reference	33 (49.3)	52 (62.7)		Reference
GC	50 (38.2)	12 (63.2)	0.07	2.67 (0. 98–7.25)	32 (47.8)	30 (36.1)	0.13	1.68 (0.86–3.25)
CC	3 (2.3)	0 (0)			2 (3)	1 (1.2)	0.56	3.15 (0.27–36.1)
IL-6 alleles								
G	206 (78.6)	26 (68.4)		Reference	98 (73.1)	134 (80.7)		Reference
C	56 (21.4)	12 (31.6)	0.21	1.69 (0.80–3.57)	36 (26.9)	32 (19.3)	0.12	1.53 (0.89–2.64)
TNF-α genotypes								
GG	109 (83.2)	13 (68.4)		Reference	54 (80.6)	68 (81.9)		Reference
GA	19 (14.5)	6 (31.6)	0.09	2.64 (0.89–7.82)	11 (16.4)	14 (16.9)	1	0.98 (0.41–2.35)
AA	3 (2.3)	0 (0)			2 (3)	1 (1,2)	0.58	2.51 (0.22–28.5)
GA+AA	22 (16.8)	6 (31.6)	0.12	2.28 (0.78–6.66)	13 (19.4)	15 (18.1)	0.83	1.09 (0.47–2.48)
TNF-α alleles								
G	237 (90.5)	32 (84.2)		Reference	119 (88.8)	150 (90.4)		Reference
A	25 (9.5)	6 (15.8)	0.25	1.77 (0.67–4.66)	15 (11.2)	16 (9.6)	0.70	1.18 (0.56–2.48)

Table 5. Comparing of risk factors for severe prognosis and risk analysis results

Risk factor	Living n (%)	Dead n (%)	Crude values		Adjusted values	
			p-value	OR (95% CI)	*p*-value	OR (95% CI)
Age (50 yr)	54 (41.2)	12 (63.2)	0.08	2.44 (0.9–6.61)	0.22	2.19 (0.61–7.83)
Leucocyte count ($\times 10^9$/l)	5 (3.8)	2 (10.5)	0.21	2.96 (0.53–16.4)	0.94	0.92 (0.08–9.90)
Platelet count ($\times 10^9$/l)	18 (13.7)	7 (36.8)	0.02	3.66 (1.27–10.5)	0.65	1.34 (0.36–4.87)
aPTT (sec)	3 (2.3)	4 (21.1)	0.005	11.37 (2.32–55.7)	0.29	2.73 (0.42–17.58)
INR (sec)	6 (4.6)	5 (26.3)	0.005	7.44 (2–27.5)	0.01	13.91 (1.79–107)
AST (U/L)	36 (27.5)	17 (89.5)	0.0001	22.4 (4.93–101)	0.001	23.3 (3.62–149)
ALT (U/L)	20 (15.3)	7 (36.8)	0.04	3.23 (1.13–9.22)	0.98	1.01 (0.26–3.88)
Fibrinogen (μg/dl)	1 (0.8)	1 (5.3)	0.24	7.16 (0.42–119)	0.81	1.59 (0.03–82.6)
TNF-α (GG)	109 (83.2)	13 (68.4)				
TNF-α (GA+AA)	22 (16.8)	6 (31.6)	0.12	2.28 (0.78–6.66)	0.28	2.12 (0.53–8.45)

type of this polymorphism (AA) was not observed in patients groups. However, it has been reported that the probability of developing DHF in patients with TNF-α-308A allele was 2.5 fold greater compared to patients without this allele, in the study. Furthermore, this allele which was reported by Fernandez-Mestre *et al*[18] to be related with hemorrhagic manifestations in DF patients was suggested as a possible risk factor of bleeding in this patient

group. In addition, an association between DHF patients and combination of TNF-α high (-308AA or AG)/IL-10 low genotype (-1082AA) has been detected[18]. Similar to this data, a significant association of TNF-α-308A allele and IL-10-1082/-819/-592ACC/ATA haplotype for DHF in Cuban patients has been detected by Perez *et al*[19]. In addition, it has been reported that high expression of allele TNF-α -308 (A allele) affected the massive increase of vascular permeability during DHF infection[19]. However, no correlation between IL-6 -174G/C polymorphism and the DHF severity in both studies were defined by Fernandez-Mestre *et al*[18] and Perez *et al*[19].

Similar to our data, any association of the putative high producing TNF-α phenotype with development of chronic HBV infection was not detected in Iran population[6]. As reported by Makela *et al*[20], we think that there is no independent effect of TNF-α and IL-6 promoter polymorphisms on the outcome and clinical course of disease.

Heterogeneity of the results obtained from several studies could be the prevalence of these polymorphisms in the studied populations. When Turkish population for genotype frequencies of both polymorphisms was compared with other populations in Western Europe, Africa, Asia, the Middle East and South America, significant differences were found[21]. Although, IL-6-174 CC genotype frequency (polymorphic type) in Turkish population (1.2%) is similar to Omani population (1.3%), but it is lower than Caucasians from N. Ireland (22%), Saudis (6%), White Americans (15.8%), Cubans (14.1%), Italians (9%), Brazilians (9.9%), Mexican Mestizas (2.5%) and, higher than Zulus (0%) and Singapore Chinese (0%)[19, 21–22]. Strikingly, heterozygous genotype (33.5%) frequency of IL-6 polymorphism in Turkish population is higher than other populations except Caucasians (48%) and White Americans (39.2), as GG genotype (wild type) frequency is lower in these populations. Genotype distributions of TNF-α-308 G/A polymorphism in Turkish population were similar to Omanis population. However, significant differences have been observed when Turkish population was compared with other populations mentioned above[19, 21–22].

Furthermore, the ethnic difference, gene-gene and/or gene-environmental interactions could be reasons for poor prognosis of multifactorial diseases such as CCHF. So, we investigated together with putative high producing TNF-α phenotype and demographic properties, and clinical symptoms and laboratory findings of patients. The effects of several demographic properties, and clinical symptoms and laboratory findings on course of the disease and mortality rate of CCHF have been evaluated in several studies[3, 23–25]. Laboratory findings like thrombocytopenia, leucocytosis or leucopoenia, elevated levels of AST and ALT, fibrinogen and INR, prolonged aPTT were found to be associated with severity of the disease and death in different studies[23–25]. Besides, advantaged age, bleeding, organ failure and hepatomegaly detected to be associated with high mortality rate of the disease in these studies[3, 23]. Our results were compatible with these findings. In addition, values of AST and INR were higher in dead cases than living cases [p=0.01, OR=13.9 (1.79–107) for INR; p=0.001, OR=23.3 (3.62–149) for AST]. So, we suggest that AST and INR may be important biomarkers for determining the risk of severity and death as a result of infection with CCHF virus. This finding was consistent with data of various studies[23-26].

In conclusion, we did not find any significant association of IL-6 -174G/C and TNF-α -308 G/A polymorphisms on the poor prognosis of CCHF and mortality in this study. To date, this is the first study that investigated the association between the course and outcome of CCHF and the polymorphisms of TNF-α -308G/A and IL-6 -174G/C. We believe that this research will be baseline for similar studies. In future, researchers should include both other cytokine and cytokine receptor gene polymorphisms for the understanding of the causes of severe clinical course of the CCHF disease. Sample size of patients, particularly number of dead individuals is a limitation in our study. Number of dead patients was higher in the year 2010. However, blood samples could be collected from only 19 patients. Some patients died before admission to the hospital. Diagnosis of these patients has been performed after their autopsies. So, samples of these patients could not be included for analysis.

Conflict of interest: None.

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