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ORIGINAL ARTICLE

LACK OF ASSOCIATION OF TUMOR NECROSIS FACTOR-α G–308A AND TRANSFORMING GROWTH FACTOR-β1 C–509T POLYMORPHISMS IN PATIENTS WITH DEEP NECK SPACE INFECTIONS

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ABSTRACT

Deep neck space infections are defined as infections that spread along the fascial planes and spaces of the head and neck. Even in the era of antibiotics, these infections can and have been potentially life-threatening conditions. The role of single nucleotide polymorphisms (SNPs) of tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) genes in deep neck infections has not been studied. Thus, the aim of this study was to investigate the distribution of the TNF-α G-308A and TGF-β1 C-509T polymorphisms in patients suffering from infections of deep neck spaces and to determine the correlation of these polymorphisms with the values of inflammation markers [C-reactive protein (CRP) and white blood cell (WBC) count]. A total of 41 patients with infections of deep neck spaces and 44 healthy controls were screened for TNF-α G-308A and TGF-β1 C-509T polymorphisms using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The distribution of the TNF-α G-308A genotype in patients did not reveal statistically significant correlation compared to con-trols ($p = 0.483$, $\chi^2 = 0.491$) as well as the distribution of the TGF-β1 C-509T genotypes ($p = 0.644$, $\chi^2 = 0.725$). The distribution of TNF-α -308 and TGF-β1 -509 alleles was not significantly different in patients compared to controls. Moreover, CRP levels and WBC counts were not associated with TNF-α G-308A and TGF-β1 C-509T promoter polymorphisms in patients with deep neck infections. In conclusion, our study suggests that the TNF-α G-308A and TGF-β1 C-509T polymorphisms are not associated with infections of deep neck spaces.

Keywords: Deep space neck infection(s); Tumor necrosis factor-α (TNF-α) polymorphism; Transforming growth factor-β1 (TGF-β1) polymorphism; C-reactive protein (CRP); White blood cells (WBC) count.

INTRODUCTION

Deep neck space infections are defined as infections that spread along the fascial planes and spaces of the head and neck [1]. They can arise from various head and neck regions. The most common etiology is pharyngitis, tonsillitis, odontogenic infections, upper respiratory infections, otitis media or trauma. The deep neck space infections produce significant morbidity and mortality, particularly when associated with the predisposing factors that impair a functional immunological response [2]. Even in the era of antibiotics, these infections have been potentially life-threatening conditions due to the airway obstruction, jugular vein thrombosis, descending mediastinitis, sepsis, acute respiratory distress syndrome and disseminated intravas-cular coagulation [2].

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Tumor necrosis factor-α (TNF-α) is a multifunctional, inducible cytosine that is produced in response to infection, inflammation and injury. Tumor necrosis factor-α can be produced by lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue [3]. It is mainly produced by the macrophages in response to activation of membrane-bound pattern-recognition molecules, which detect common bacterial cell surface products such as polysaccharides, carbohydrates and lipopolysaccharides (LPS). It is the main mediator in response to Gram-negative bacteria and concentration of TNF-α correlates with the amount of bacteria and the phase of inflammation [4].

Transforming growth factor-β1 (TGF-β1) is a pleio-tropic cytokine with a variety of effects on a wide range of cells in the immune system, playing an important role in cell differentiation, growth, matrix formation, and regulation of immune and inflammatory responses [5]. It is also a very potent stimulator of monocyte, lymphocyte, neutrophil and fibroblast migration [6].

The genetic control of inflammatory response in humans has been extensively studied, including the investigation of TNF-α and TGF-β1 responses. Numerous studies have shown that the variations in production and activity of cytokines influence the susceptibility and/or resistance to a range of infectious agents, autoimmune diseases, cancer and other disorders [7]. Differences in the production of cytokines between individuals are often caused by single nucleotide polymorphisms (SNPs) in the promoter or coding regions of cytokine genes that directly affect the transcription and synthesis of mRNA [8]. A biallelic polymorphism at the position –308 within the promoter region of the TNF-α gene is one of the most investigated. The presence of the polymorphic TNF-α –308A allele is considered to be associated with the higher TNF gene transcription and TNF-α overproduction [9]. This substitution leads to 2- to 3-fold higher transcriptional activity of the TNF-α upon stimulation with bacterial LPS [10].

The TGF-β1 gene also has several polymorphisms, including C-988A, G-800A and C-509T. The cytosine (C) to thymine (T) base exchange at position –509 relative to the first major transcription start site of the TGF-β1 gene was found to be differentially related to transcription factor binding to the TGF-β1 promoter, transcriptional activity of TGF-β1, and TGF-β1 plasma concentration [11].

Genetic variations within the cytokine genes may be critical in understanding individual predisposition and susceptibility to different clinical conditions. To the best of our knowledge, there are no available studies examining the distribution of TNF-α G-308A and TGF-β1 C-509T polymorphisms in patients suffering from deep neck space infections. Thus, the aim of this study was to analyze the distribution of these polymorphisms and their correlation with the values of inflammatory markers [C-reactive protein (CRP) and white blood cell (WBC) count] in patients suffering from infections of deep neck spaces.

MATERIALS AND METHODS

Patients and Controls. Blood samples were collected from 41 patients admitted at the Department of Maxillo-facial Surgery, Dental Clinic, Niš, Serbia. The patients with deep neck infections were classified into three groups: abscess, phlegmon and others (*e.g.,* cellulitis, suppurative parotitis, *etc.*), where the particular diagnoses that were included in "others" did not have a sufficient number of patients to perform statistical tests. Forty-four randomly selected healthy individuals, without known acute or chronic disease, were included in the study as a control group. An informed consent was obtained from all participants and the study was approved by the Ethical Committee of the Medical Faculty, University of Niš, Niš, Serbia. At the moment of admission to the hospital, venous blood samples were obtained from the median cubital vein and collected into EDTA vaccutainer tubes. Two-hundred microliters of blood were used for DNA isolation and the rest for biochemical analysis. An automatic hematology analyzer (MEK 6318K; Nihon Kohden, Tokyo, Japan) was used for WBC count determination. The CRP levels were measured using nephelometric immunoassay (Dade Behring, Marburg, Germany).

DNA Isolation and Polymerase Chain Reaction Amplification. Genomic DNA was isolated from whole blood samples using QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The biallelic polymorphisms within TNF-α and TGF-β1 genes were determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Polymerase chain reaction was performed in a final volume of 25 μL containing 20 ng of DNA, 12.5 μL KAPA2G Fast HotStart

Table 1. Primer sequences, restriction enzymes and size of fragments generated by the TNF-α G-308A and TGF-β1 C-509T gene polymorphisms.

Polymorphism	Primer Sequences (5'>3')	Restriction Enzyme	Allele Determination
TNF-α G-308A	F: AGG CAA TAG GTT TTG AGG GCC AT R: ACA CTC CCC ATC CTC CCT GCT	*Nco*I	G 97+20 bp A 117 bp
TGF-β1 C-509T	F: GGA GAG CAA TTC TTA CAG GTG R: TAG GAG AAG GAG GGT CTG TC	*Hpy*F31	C 74+46 bp T 120 bp

ReadyMix (Kapa Biosystems Inc, Boston, MA, USA) and 20 pmol of each primer. Primer sequences used in this study are summarized in the Table 1. The PCR conditions were as follows: initial denaturation at 95°C for 2 min., followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds, ending with a final extension at 72°C for 1 min. The PCR products were electrophoresed on a 2.0% agarose gel, stained with ethidium bromide and visualized under UV light.

The amplification products were digested using *Nco*I and *Hpy*F3I restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany). Restriction enzyme digestion was carried out at 37°C overnight and analyzed by 8.0% polyacrylamide gel electrophoresis (PAGE). The gel was stained with ethidium bromide and visualized under UV light (Figures 1 and 2). The interpretation of the obtained results was performed according to Table 1.

Genotype analyses were performed by two independent researchers. After the polymorphic alleles were established to be homozygous, the PCR-RFLP was repeated in order to confirm the obtained results.

Statistical Analyses. The allele and genotype frequencies were determined in patients and controls. They were compared with the values predicted by the Hardy-Weinberg equilibrium using the χ^2 test. The two-tailed Fisher's test was used when the number of expected cases was small. Genetic risk magnitudes (effect size) were estimated by calculating odds ratios (ORs) with 95% confidence intervals (95% CI). C-reactive protein levels and WBC counts were expressed by a median (25th to 75th percentiles). The correlation of TNF-α -308 and TGF-β1 -509 genotypes with CRP and WBC count values were determined using the Mann-Whitney U test. One-way analysis of variance (ANOVA) was used to compare the mean CRP levels and WBC counts between the groups. Probability values less than 0.05 (p <0.05) were considered statistically significant. Statistical analyses were performed using the SPSS version 13.0 statistical software package (SPSS Inc, Chicago, IL, USA).

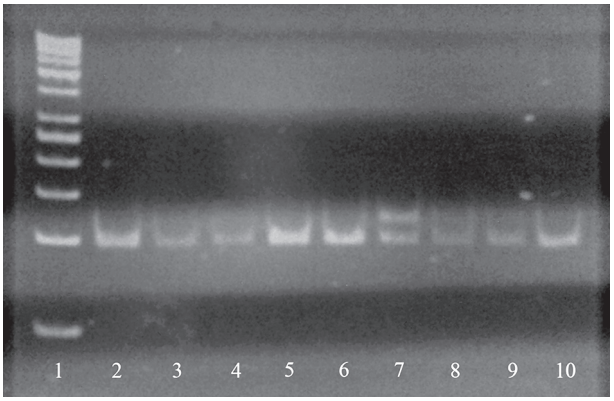

Figure 1. The TNF-α PCR products after RFLP, stained with ethidum bromide. Lane 1: ladder; lanes 2-6, 8-10: GG; lane 7: GA.

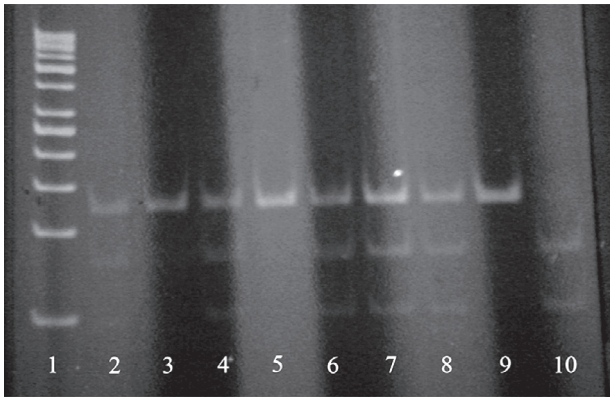

Figure 2. The TGF-β1 PCR products after RFLP, stained with ethidum bromide. Lane 1: ladder; lanes 2, 4, 6-8: CT; lanes 3, 5 and 9: TT; lane 10: CC.

RESULTS

Forty-one patients and 44 unrelated controls were involved in this study. The demographic characteristics of the study groups are summarized in Table 2.

Genotype frequencies for the SNPs in the study groups were in Hardy-Weinberg equilibrium (p >0.05). As the TNF-α -308 AA genotype was present in only a small number of subjects (only one in the control group and two in the patients), it was analyzed together with individuals heterozygous for the TNF-α -308 polymorphism. The observed TNF-α -308 genotype distribution in the patients' group did not show significant differences compared to controls (Table 3). Moreover, no differences in the distribution of TNF -308G and TNF -308A alleles were observed between the patient and control groups (Table 4). The genotype and allele distribution of the TGF-β1 C-509T gene polymorphism did not show significant differences compared to controls (Tables 3 and 4). Also, no association of the particular genotype or allele of the TNF-α G-308A and TGF-β1 C-509T polymorphisms was obtained after the classification of the samples by diagnosis (Table 5).

Furthermore, in order to evaluate the common association of polymorphic alleles, we investigated the association of the combination of high producing TNF -308A and TGF -509T alleles. The data regrouping was as follows: A^+/T^+ (high TNF-α/high TGF-β1), A^+/T^- (high TNF-α/low TGF-β1), A^-/T^+ (low TNF-α/high TGF-β1), A^-/T^- (low TNF-α/low TGF-β1). However, no statistically significant differences were observed ($\chi^2 = 1.069$, df = 3, $p = 0.784$).

Table 2. Characteristics of the study groups.

	Patient Group (n = 41)	**Control Group (n = 44)**
Males	22 (53.7%)	21 (47.7%)
Females	19 (46.3%)	23 (52.3%)
Age at the beginning of the study[a]	47.8 ± 4.54	48.5 ± 16.73
Clinical diagnosis: Abscess	19 (46.34%)	
Phlegmon	12 (29.27%)	
Others[b]	10 (24.39%)	

[a] Data are presented as mean ± standard deviation.
[b] Some diagnoses were included in "others" as there were an insufficient number of patients to perform statistical tests.

Table 3. Genotype distribution of the TNF-α G-308A and TGF-β1 C-509T polymorphisms in patient and control groups.

Gene Polymorphism	**Genotype**	**Controls n (%)**	**Patients n (%)**	χ^2	***p* Value**	**OR**	**95% CI**
TNF-α G-308A	G	29 (65.9)	24 (58.5)				
	GA+AA	15 (34.1)	17 (41.5)	0.491	0.483	1.369	0.658-3.302
TGF-β1 C-509T	CC+CT	35 (79.5)	32 (78.05)				
	TT	9 (20.5)	9 (21.95)	0.028	0.866	1.094	0.386-3.097
	CC	13 (29.5)	9 (21.95)				
	CC+TT	31 (70.5)	32 (78.05)	0.638	0.424	1.491	0.558-3.985

OR: odds ratio; 95% CI: 95% confidence interval.

Table 4. Allele frequencies of the TNF-α G-308A and TGF-β1 C-509T gene polymorphisms in the patient and control groups.

Allele	**Controls n (%)**	**Patients n (%)**	χ^2	***p* Value**	**OR**	**95% CI**
G	72 (81.8)	63 (76.8)				
A	16 (18.2)	19 (23.2)	0.646	0.421	1.357	0.644-2.862
C	48 (54.5)	41 (50.0)				
T	40 (45.5)	41 (50.0)	0.352	0.553	1.200	0.657-2.193

OR: odds ratio; 95% CI: 95% confidence interval.

Table 5. Genotype distribution and allele frequencies of the TNF-α G-308A and TGF-β1 C-509T in patients with deep neck infections.

Gene Polymorphism	Genotype/ Allele	Abscess *n* (%)	Plegmon *n* (%)	Others *n* (%)	p_1	p_2	p_3
TNF-α G-308A	GG	11 (57.9)	6 (50.0)	7 (70.0)			
	GA + AA	8 (42.1)	6 (50.0)	3 (30.0)	0.724	0.694	0.415
	G	29 (76.32)	17 (70.83)	17 (85.0)			
	A	9 (23.68)	7 (29.17)	3 (15.0)	0.767	0.515	0.306
TGF-β1 C-509T	CC	5 (26.32)	2 (16.67)	2 (20.0)			
	CT + TT	14 (73.68)	10 (83.33)	8 (80.0)	0.676	1.000	1.000
	C	20 (52.63)	11 (45.83)	10 (50.0)			
	T	18 (47.37)	13 (54.17)	10 (50.0)	0.795	1.000	1.000

p_1: Abscess *vs.* phlegmon; p_2: abscess *vs.* others; p_3: phlegmon *vs.* others.

Table 6. The CRP levels and WBC counts in relation to the TNF-α G-308A and TGF-β1 C-509T genotypes in patients with deep neck infections.

	TNF-α G-308A			TGF-β1 C-509T			
	GG	GA + AA	*p* Value	CC	CT	TT	*p* Value
CRP	177.70 (89.00-238.07)	108.14 (26.30-311.80)	0.628	200.00 (139.00-200.00)	85.10 (26.30-135.50)	155.20 (64.97-329.50)	0.295
WBC	15.35 (12.22-17.10)	13.80 (11.20-20.60)	0.295	15.27 (15.27 ± 0.25)[a]	12.85 (10.70-23.10)	15.25 (10.87-18.05)	0.692

Data are presented as median (25th to 75th percentiles).
[a] Data are presented as mean ± standard deviation.

C-reactive protein levels and WBC counts, as well as their relation to TNF-α G-308A and TGF-β1 C-509T genotypes were determined in patients with deep neck space infections. The CRP levels were found to be 5- to 60-fold over the base line. The obtained results show no correlation of CRP levels and WBC counts with TNF-α G-308A and TGF-β1 C-509T genotypes (Table 6). Furthermore, after the classification of the patients by diagnosis, neither CRP levels ($p = 0.699$) nor WBC counts ($p = 0.787$), showed significant difference between the groups.

DISCUSSION

Deep neck infections are less common in the antibiotic era but they often have a rapid onset and can progress to life-threatening complications, especially in the elderly and patients with systemic diseases associated with impaired functional immunologic response. The most common source of inflammation of deep neck spaces in adults are odontogenic infections with the involvement of the submandibular space [12].

The presence of the functional polymorphisms in cytokine genes affect cytokine expression, and thus may have an important role in the genetic regulation of the inflammatory response and resistance or susceptibility to infections [13]. The gene for TNF-α is located in chromosome 6 (region p21.3) within the class III region of the major histocompatibility complex. The substitution of guanine (G) with adenine (A) at the -308 site of the TNF-α gene generates two alleles, TNF -308G and TNF -308A. The less common TNF -308A allele is considered to be associated with higher TNF gene transcription and TNF-α overproduction [9]. A number of studies indicate that the TNF-α G-308A polymorphism is associated with the higher susceptibility for a variety of inflammatory and autoimmune diseases [14-18]. In oral and maxillofacial pathology, the TNF-α G-308A polymorphism has been studied in patients with burning mouth syndrome, aph-thous stomatitis and

periodontal disease. Some investigators observed a higher TNF-α production in the carriers of the TNF -308A allele, while others found no functional significance of this SNP [19,20].

To the best of our knowledge, there are no reported studies concerning the association of the TNF-α G-308A polymorphism with infections of deep neck spaces. However, our study did not confirm significant differences in the genotype and allele frequency distribution of the patient and control groups. Even though it is well known that TNF-α is a potent chemotactic factor for WBCs, our study did not show any association of the TNF-α -308 polymorphism with WBC count. Moreover, proinflammatory response of TNF-α results in its increased secretion, and releasing of the messenger cytokine, interleukin-6 (IL-6), that stimulates the liver to secrete CRP, which is reliable marker of the acute phase response to infectious burdens and/or inflammation [21]. In healthy adults, the TNF AA genotype is associated with increased plasma CRP levels in Caucasian and Black men and in Caucasian women, suggesting that this polymorphism contributes to variability in plasma CRP levels [22]. Our results showed a 5- to 60-fold over the baseline rise of CRP levels in patients with deep neck space infections, but without significant differences in CRP values in the presence of the TNF -308A allele. This can partially be explained by the very low number of homozygous TNF -308A allele carriers, reflecting the low frequency of the AA genotype in this study population. These results are in accordance with those previously reported in the literature that approximately 60.0-70.0% of the Caucasian populations are homozygous for the wild type TNF -308G allele, 30.0-40.0% are heterozygous, and only 1.5-3.0% are homozygous for the variant TNF -308A allele [23].

The TGF-β1 polymorphism provides chemotactic stimuli for leukocyte migration, but in contrast to its che-motactic effects, it also shows anti-inflammatory effects [24,25]. The TGF-β1 gene is located on chromosome 19 (q13.1-13.3). A C>T SNP at position -509 relative to the first major transcription start site was found to be differentially related to transcription factor binding to the the TGF-β1 promoter, transcriptional activity of TGF-β1, and TGF-β1 plasma concentration [11]. This polymorphism was previously studied in asthma, chronic obstructive pulmonary disease, hepatocelluar and gastric cancer [26-29]. In oral pathology, the TGF-β1 C-509T promoter polymorphism was mostly studied in chronic periodonitis [30,31]. To the best of our knowledge, this is the first study to examine the association of the TGF-β1 C-509T polymorphism with deep neck infections. Our results suggest that this polymorphism is not associated with deep neck space infections. Additionally, no association of the TGF-β1 C-509T polymorphism with WBC counts and CRP levels was observed in patients with deep neck space infections.

This study also showed no association of the TNF-α G-308A and TGF-β1 C-509T polymorphisms with certain diagnoses such as abscess or phlegmon. No difference between CRP levels and WBC counts was obtained after the classification of the samples by diagnosis.

Since the cytokines act in a highly complex coordinated network, it would be of great importance to investigate the common influence of the genetic polymorphisms that regulate their production. Particularly, TGF-β1 is known to have a potent immunosuppressive activity, downregulating the transcription of other proinflammatory cytokines, including TNF-α [30]. In order to evaluate the common association of polymorphic alleles, we have investigated the association of the combination of high producing TNF -308A and TGF -509T alleles. However, no statistically significant differences were observed. Generally, the discrepancies in observed results, besides the genetic heterogeneity of the study populations, might also be explained by population stratification and population bias.

In conclusion, this is the first study examining the association of the SNPs of the TNF-α and TGF-β1 genes in patients with deep neck infections. The present study did not confirm the specific role of the TNF-α G-308A and TGF-β1 C-509T polymorphisms in patients with the infections of deep neck spaces. However, further studies are needed to examine genetic markers that can be used for following the disease progression and early identification of individuals at high risk of developing complications.

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