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Source / Izvornik: **Medical science monitor, 2009, 15, 361 - 365**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:184:337341

© Med Sci Monit, 2009; 15(7): CR361-365
PMID: 19564826

Received: 2007.12.13
Accepted: 2008.02.18
Published: 2009.07.01

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
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F Literature Search
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Source of support: Departmental sources

Summary

Background: Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine involved in the pathogenesis of various inflammatory and malignant diseases. Previous studies investigating the role of the TNF-α gene polymorphisms in lung cancer have generated contradictory results. The present study investigated whether the TNF-α-308 and TNF-α-238 polymorphisms are associated with risk and/or severity of disease in Croatian lung cancer patients. This is the first study in a Caucasian population to analyze the influence of these two polymorphisms on multiple types of lung cancer.

Material/Methods: In a case-control study, lung cancer patients (n=230) and appropriate age- and sex-matched controls (n=230) were genotyped by the polymerase chain reaction/restriction fragment length polymorphism method. Allele and genotype frequencies were estimated by gene counting. The chi-squared test was used to compare the observed numbers of different TNF-α genotypes for the population with those predicted by Hardy-Weinberg equilibrium. Differences in genotype and allele distributions in the patient and control groups were analyzed for statistical significance using the chi-squared test or Fisher's exact test as appropriate.

Results: There were no significant differences in the genotype and allele frequencies for the TNF-α-308 and TNF-α-238 polymorphisms between lung cancer patients and controls. Furthermore, no association between the genotypes and different stages of lung cancer was detected.

Conclusions: This study indicates that the TNF-α-308 and TNF-α-238 polymorphisms do not influence susceptibility to or severity of lung cancer in a Croatian population.

key words: **lung cancer • TNF-α polymorphism • tumor type • tumor stage • Caucasian population • cancer susceptibility**

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=869711
Word count: 1681
Tables: 3
Figures: –
References: 31

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BACKGROUND

Lung cancer is a multifactorial disease with a complex interplay of environmental and genetic factors contributing to its progression. The cause of the most common and fatal malignant tumors in Europe [1], lung cancer ranks as the most prevalent cause of mortality in men and the second most prevalent in women in Croatia [2]. Individual cancer risk may be mediated by physical factors within the airways as well as individual responses to environmental and occupational mutagens [3].

Tumor necrosis factor-α (TNF-α) is a potent pro-inflammatory cytokine that functions as a central mediator of the immune response in a wide range of immune-inflammatory and infectious diseases [4]. In addition to its role in inflammation and as a significant modulator of airway inflammation [4], TNF-α has also been recognized as an important factor in cancer development and spread. Previous studies have shown that TNF-α has antitumor effects, but it has also been shown to be tumorigenic both *in vitro* and *in vivo* [5]. TNF-α alone can induce apoptosis in human cancer cell lines [6], but increasing evidence suggests that TNF-α may also promote the development and spread of cancer. Increased levels of TNF-α were detected in the serum of cancer patients and have been associated with an adverse disease outcome [7–9].

Cytokine promoter polymorphisms, including TNF-α-308 and TNF-α-238, are associated with altered protein levels and/or rates of transcription [8]. Several polymorphisms have been associated with a higher production of TNF-α, including the G/A transitions at positions -308 and -238 [10], and these polymorphisms have been the subject of many studies investigating their functional significance [11]. To our knowledge, only two studies thus far have investigated the role of TNF-α polymorphisms in the susceptibility to and severity of lung cancer. In the first study the TNF-α-308 polymorphism, along with polymorphisms in TNF-β, interleukin 6 (IL-6), and interleukin 10 (IL-10), were analyzed in all types of primary lung cancer in 117 German patients [8]. No correlation was observed between the TNF-α-308 polymorphism and lung cancer susceptibility. In the second study the TNF-α-308 and TNF-α-238 polymorphisms were investigated in 202 Chinese patients with non-small-cell lung cancer (NSCLC) [12]. In contrast to the previous finding, this study found a significant association between the -308 G/A and -238 G/A polymorphisms in the TNF-α promoter region and lung cancer susceptibility. Moreover, these two polymorphisms were shown to be related to the severity of disease. The -308 A allele positively correlated with lung cancer development and progression, whereas the -238 A allele had a protective impact on the development of lung cancers.

The apparent discrepancy in the previous reports led us to investigate further the role of TNF-α polymorphisms in the development of lung cancer. In this study we conducted an analysis of a Croatian patient population to determine whether the TNF-α-308 and -238 polymorphisms contribute to lung cancer susceptibility and/or modify the disease course.

MATERIAL AND METHODS

A total of 230 primary lung cancer patients (163 males and 67 females) of the Department of Pulmonology, Clinical Hospital Center, Rijeka, Croatia, were recruited prospectively into the study from 2006 to 2007. Patient data, including patient demographics and lifestyle, past medical history, treatment, and survival, were collected by interview and entered into a custom-built database. The histological determination was performed according to the WHO classification method (WHO, 1999) [13] and the classification of tumor stages according to the TNM system (Mountain, 1997) [14]. The control group consisted of 230 unrelated age- and sex-matched healthy blood donors with no family history of lung cancer.

The Ethics Committee of the School of Medicine of the University of Rijeka approved the study and written informed consent was obtained from each subject. The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Genomic DNA was extracted from peripheral blood lymphocytes according to standard protocols. The analysis of TNF-α gene polymorphisms was performed by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) analysis under the conditions described by Shih et al. [12].

Allele and genotype frequencies were estimated by gene counting. The χ^2 test was used to compare the observed numbers of the different TNF-α genotypes for a population with those predicted by Hardy-Weinberg equilibrium. Group differences in genotype and allele distribution in the patient and control groups were analyzed for statistical significance using the χ^2 test or Fisher's exact test as appropriate. Odds ratios (*OR*) and their 95% confidence intervals (*CI*) were calculated to evaluate the effects of different genotypes/alleles. A p value <0.05 was considered statistically significant.

Based on the number of 230 patients and 230 control subjects, the statistical power was 80% to identify a 1.5-fold increase in the frequency of TNF-α-308 heterozygosity (26% carriers in the control subjects). For TNF-α-238 (7% carriers in the control subjects), the statistical power was 80% to identify a 2.2-fold increase in frequency of heterozygosity.

RESULTS

Of the 230 patients with lung cancer, 201 (87%) had non-small-cell lung cancer (NSCLC). This group included 144 males and 57 females and the median age was 67 years (range: 31–92 years). Of the NSCLC group, 67 (29%) patients had adenocarcinomas (AD), 113 (49%) had squamous carcinomas (SQ), and 21 (9%) were diagnosed with other types of carcinomas (3 large-cell carcinomas and 18 with an undetermined type of non-small-cell carcinoma). The small-cell lung cancer (SCLC) group consisted of 29 (13%) patients, 19 males and 10 females, with a median age of 60 years (range: 45-88 years). A history of smoking was reported in 86% of the NSCLC group and 93% of the SCLC group. Forty-five (20%) patients were classified with stage I or II lung cancer and 185 (80%) with stage III or IV.

Analyses of the genotype and allele frequencies of the TNF-α-308 and TNF-α-238 polymorphisms in the lung cancer patients and controls are presented in the tables. No sig-

Table 1. Allele frequencies of the TNF-α-308 and TNF-α-238 gene polymorphisms in lung cancer patients and controls.

	TNF-α (308 G/A) polymorphism			TNF-α (238 G/A) polymorphism		
	Allele G (%)	Allele A (%)	p	Allele G (%)	Allele A (%)	p
Controls (n=230)	85.9	14.1		96.5	3.5	
Total lung cancer (n=230)	84.8	15.2	0.64	97.0	3.0	0.71
NSCLC (n=201)	84.3	15.7	0.59	97.0	3.0	0.83
SCLC (n=29)	87.9	12.1	0.82	96.5	3.5	0.99

NSCLC – non-small-cell lung cancer; SCLC – small-cell lung cancer.

Table 2. Association between the TNF-α-308 polymorphism and clinicopathological parameters of the subjects.

Characteristics	Genotypes			Total	p	OR (95% CI)
	AA (%)	GA (%)	GG (%)			
Controls	6 (3)	53 (23)	171 (74)	230		
Lung cancer	9 (4)	52 (23)	169 (73)	230	0.83	1.05 (0.68–1.62)
Tumor type						
AD	3 (4)	12 (18)	52 (78)	67	0.59	0.84 (0.42–1.66)
SQ	4 (4)	30 (27)	79 (70)	113	0.38	1.25 (0.73–2.12)
OC	1 (5)	5 (24)	15 (71)	21	0.77	1.16 (0.38–3.38)
NSCLC total	8 (4)	47 (24)	146 (73)	201	0.69	1.09 (0.71–1.68)
SCLC	1 (4)	5 (17)	23 (79)	29	0.56	0.76 (0.26–2.08)
Tumor stage						
I + II	2 (4)	8 (18)	35 (78)	45	0.47	
III + IV	7 (4)	44 (24)	134 (72)	185		

AD – adenocarcinoma; SQ – squamous cell carcinoma; OC – other carcinoma; NSCLC – non-small-cell lung cancer; SCLC – small-cell lung cancer; OR – odds ratio (95% confidence interval); GA plus AA vs. GG.

nificant deviations from the predicted Hardy Weinberg proportions were observed in either the patients (TNF-α-308: p=0.46, TNF-α-238: p=0.85) or controls (TNF-α-308: p=0.78, TNF-α-238: p=0.86). No significant differences were observed in G and A allele frequencies of the TNF-α-308 and TNF-α-238 gene polymorphisms in the lung cancer patients and controls (p=0.64 and p=0.71, Table 1).

The distribution of the TNF-α-308 G/A genotypes in the lung cancer patients was similar to that in the healthy subjects (p=0.83, Table 2). Due to the low frequency of patients and controls homozygous for the A allele, all carriers of the A allele, both homozygous and heterozygous, were grouped for further analysis. No significant differences (p=0.47) were observed in tumor stage between patients carrying the A allele and those homozygous for the G allele.

Regarding the TNF-α-238 G/A genotype distributions, no significant differences between controls and lung cancer patients were found (p=0.71, Table 3). We also observed no significant difference in tumor stage among the patients with TNF-α-238 GA genotypes and those with TNF-α-238 GG genotypes (p=0.28).

Similar results were obtained when overall NSCLC lung cancer patients or the subgroups (AD, SQ, OC) were compared separately to the healthy controls as when SCLC cancer patients were compared to the controls (Tables 1–3). Also, separating lung cancer patients by gender, familiarity, or smoking status did not generate any statistically significant differences in allele and genotype distributions (data not shown).

Discussion

In this study we found no association between either TNF-α-308 or TNF-α-238 biallelic polymorphisms and lung cancer, and thus no evidence that these polymorphisms contribute to disease susceptibility or severity. Also, when tumor types were considered in the analysis of allelic and genotype frequencies, no significant differences in the risk of any type of lung cancer were apparent.

These results are in line with a previous study which demonstrated a lack of association between the TNF-α-308 polymorphism and lung cancer in German patients [8]. Seifart et al. previously suggested a connection between the IL-10-1082-G

Table 3. The association between the TNF-α-238 polymorphism and clinicopathological parameters of the subjects.

Characteristics	Genotypes			Total	p	OR (95% CI)
	AA (%)	GA (%)	GG (%)			
Controls	0 (0)	16 (7)	214 (93)	230		
Lung cancer	0 (0)	14 (6)	216 (94)	230	0.71	0.87 (0.39–1.93)
Tumor type						
AD	0 (0)	5 (7)	62 (93)	67	0.89	1.08 (0.33–3.31)
SQ	0 (0)	5 (4)	108 (96)	113	0.36	0.62 (0.19–1.86)
OC	0 (0)	2 (10)	19 (90)	21	0.45	1.41 (0.00–7.16)
NSCLC total	0 (0)	12 (6)	189 (94)	201	0.94	0.97 (0.44–2.15)
SCLC	0 (0)	2 (7)	27 (93)	29	0.67	0.99 (0.00–4.88)
Tumor stage						
I + II	0 (0)	4 (9)	41 (91)	45	0.28	
III + IV	0 (0)	10 (5)	175 (95)	185		

AD – adenocarcinoma; SQ – squamous cell carcinoma; OC – other carcinomas; NSCLC – non-small-cell lung cancer; SCLC – small-cell lung cancer; OR – odds ratio (95% confidence interval); GA plus AA vs. GG.

allele and SCLC. Although the mechanism(s) of this correlation is still undetermined, it is known that inflammatory events may lead to local respiratory conditions which may result in increased susceptibility to lung cancer.

Shih et al. [12] provided the first report of an association between the TNF-α-308 and TNF-α-238 gene polymorphisms and risk of NSCLC in a Chinese population. Although the methods and the sample size of patients were similar, our findings in a Caucasian population did not confirm these results. The apparent inconsistency in the results regarding the role of the TNF-α polymorphisms might be due to differences in lung cancer etiology for patients of different ethnic origin.

Significant associations between the TNF-α-308 polymorphism and increased susceptibility to renal cell carcinoma, non-Hogkin's lymphoma, hepatocellular carcinoma, gastric carcinoma, bladder cancer, melanoma, and oral carcinoma have been described [7,9,11,15–18]. In other investigations the TNF-α-308 polymorphism was not associated with gastric carcinoma, myeloma, basal cell carcinoma, cervical cancer, or breast cancer [10,19–26]. Similarly, the TNF-α-238 polymorphism was shown not to be associated with increased risk for gastric cancer, breast cancer, cervical cancer, and esophageal carcinoma [17,25,27–29], but other investigations, in contrast, showed a protective function of TNF-α-238 against gastric cancer, cervical cancer, colorectal cancer, and renal cell carcinoma [30].

Why these results differ remains to be determined, and an important consideration is ethnic variability as a potentially critical factor. The genotype and allele frequencies of the TNF-α-308 and TNF-α-238 gene polymorphisms in different populations vary dramatically according to ethnicity [25,31]. Another possible reason for the above discrepancies might be due to differences in study design and data analysis or to type I/type II statistical errors. Although our study had 80% power to detect a twofold increase in the frequency of the TNF-α-238 A allele, the number of patients with the TNF-α-238 GA genotype was too small (n=14) to permit firm conclusions on the effect of TNF-α-238 polymorphism and on the lung cancer subgroup analysis. Further studies in a larger series of patients from populations with different genetic backgrounds are necessary to clarify the potential role of TNF-α gene polymorphisms in lung cancer susceptibility.

Conclusions

The present study showed that the TNF-α-308 and TNF-α-238 polymorphisms do not influence susceptibility to lung cancer or affect its clinical course in a Croatian population.

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