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# ASSOCIATION OF TNF-R2 (676T>G) SINGLE NUCLEOTIDE POLYMORPHISM WITH HEAD AND NECK CANCER RISK IN THE SERBIAN POPULATION

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*Abstract* - Tumor necrosis factor (TNF) which exerts its effects through two different receptors known as TNF-R1 and TNF-R2, is a major proinflammatory cytokine involved in the pathogenesis of different types of tumors. We have investigated whether polymorphisms in TNF-alpha (-308G>A), TNF receptor 1 (36A>G) and TNF receptor 2 (676T>G) genes modulate the susceptibility for oral squamous cell carcinomas (OSCCs) and basal cell carcinomas (BCCs) of the skin, two frequent types of head and neck cancers. Genotyping was done on 50 OSCC patients, 50 BCC patients and 60 healthy individuals, using PCR/RFLP. A significant difference in genotype and allele frequencies was found between patients and controls for the TNF-R2 polymorphism, in both OSCC and BCC. There was no statistically significant difference between patients and controls for TNF-alpha and TNF-R1 polymorphisms. Carriers of G allele had an approximately 2.5- and 5-fold higher risk for OSCC and BCC, respectively, in the Serbian population.

*Key words:* Gene polymorphisms, TNF-alpha, TNF-R1, TNF-R2, head and neck cancer

## INTRODUCTION

Head and neck cancers are among the most common malignancies and their overall incidence, especially those of the larynx and the oral cavity, is increasing worldwide, in particular among young people (Parkin et al., 2005). Localizations of these malignancies include oral and nasal cavity, pharynx, paranasal sinuses, larynx, salivary glands and sun-exposed areas of the skin of head and neck (Burtness, 2002). Squamous cell carcinomas of the oral cavity (OSCC) account for approximately 500,000 new cases worldwide, making it the 6th most common cancer type (Parkin et al., 2005). OSCCs are considered aggressive tumors with marked tendency to recurrences and metastases. The overall 5-year survival rate is just 50% (van Oijen and Slootweg, 2000). Almost 85% of patients with OSCC are alcohol or tobacco abusers, or both (Williams, 2000).

Basal cell carcinomas (BCCs) are nonmelanoma skin cancers that develop in sun-exposed areas and therefore a large proportion, around 74%, occurs in the head and neck (HNBCCs) region (Jung et al., 2010). HNBCCs are non-aggressive tumors with slow growth and low recurrence rates (Ting et al., 2005). Local destruction may occur if left untreated or incompletely removed (Ko et al., 1992).

Although there are many different factors and mechanisms involved in tumor pathogenesis and invasion, among others the increased expression of several cytokines has been observed during the development of head and neck carcinomas (Woods et al., 1998).

Though TNF-alpha has a well-established antineoplastic role, the dysregulation of gene expression in the TNF-TNF receptor superfamily has been involved in various human cancers, through the stimulation of DNA damage, angiogenesis, tumor growth, invasion and metastasis (Mantovani, 2005).

TNF-alpha expression is influenced by polymorphisms in the TNF-alpha gene promoter (Raabe et al., 1998). A special emphasis has been put on the (-308G>A) polymorphism. Guanine at position -308 defines the common TNF1 allele and adenine the less common TNF2 allele. The A allele of TNF-alpha at position -308 polymorphism has been associated with increased TNF-alpha production (Kroeger et al., 1997). TNF2 allele has been shown to correlate with unfavorable outcome in myeloid malignancies (Davies et al., 2000).

TNF-alpha exerts its effects via two members of the TNF receptor superfamily, tumor necrosis factor receptors 1 and 2 (TNF-R1, TNF-R2). The structural similarity between the two TNF receptors is limited to their extracellular domains (Tartaglia and Goeddel, 1992). TNF-R1 has a role in cell death as well as in normal immune regulation, whilst TNF-R2 lacks a death domain (DD) and thus cannot stimulate the apoptotic process. However, TNF-R2 interference with programmed-cell-death cannot be ruled out. The function of TNF-R2 should not be underestimated, particularly because this receptor shows a restricted but inducible expression (Carpentier et al., 2004). Functional SNPs in TNF-alpha receptors genes have been described as well. Association studies dealing with polymorphisms in the genes responsible for the control of immune and inflammatory processes, in patients with head and neck tumors, are extremely scarce and mainly limited to Asian populations. This prompted us to examine the patterns of variation in three SNPs in the following candidate genes: TNF-alpha, TNF-receptor 1 and TNF-receptor 2, and to establish whether any of the polymorphisms may represent a risk factor for the development of two very frequent types of head and neck tumors, characterized by quite different clinical behavior, one aggressive (OSCC) and the other relatively benign (BCC).

## MATERIALS AND METHODS

### *Patients*

The study included 100 patients, 50 with OSCC (mean age 69 years, 35 males, 15 females) and 50 with BCC (mean age 73 years, 20 males, 30 females) treated at the Clinic of Maxillofacial Surgery, School of Dentistry, University of Belgrade, as well as 60 healthy individuals, age matched, all of Serbian ethnicities. The study protocol was approved by the institutional Ethical Committee. In the patients group, DNA was isolated from the tissue of histologically negative tumor margins, whilst in the control group DNA was isolated from buccal swabs. For both isolations a QIAmp DNA Mini Kit (Qiagen,GmbH, Germany) was used, as recommended by the manufacturer.

### *Genotyping*

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to detect the TNF-alpha (-308G>A), TNF-R1 (36A>G) and TNF-R2 (676T>G) substitution polymorphisms.

The region of the TNF-alpha gene containing the -308 position was amplified using the primers (0.25μM) forward: 5'-AGG CAA TAG GTT TTG AGG GCC AT-3'; and reverse: 5'-ACA CTC CCC ATC CTC CCT GCT-3'. A 117 bp PCR product was generated using the following amplification conditions: 94˚C for 3 min, followed by 35 cycles at 94˚C for 60 s, 60˚C for 60 s and 72˚C for 60 s, with a final extension step at 72˚C for 10 min. NcoI restriction enzyme (MBI Fermentas, Lithuania) digestion was performed at 37˚C overnight (1 unit NcoI and 10 μl PCR products). The genotypes were then assessed by PAA gel electrophoresis. The fragments were as follows: 97 and 20 bp fragments in GG homozygous individuals, three fragments 117bp, 97bp and 20bp in GA heterozygous individuals and only single 117bp fragment in AA homozygous individuals.

The region of the TNF-R1 gene containing the +36 position was amplified using the primers (0.2μM) forward: 5'-GAC CCC AAA TGG GGG AGT GAG AGG-3'; reverse: 5'-ACC AGG CCC GGG CAG GAG AG-3'. A 183 bp PCR product was generated using the following amplification conditions: 94˚C for 3 min, followed by 35 cycles at 94˚C for 60 s, 65˚C for 60 s and 72˚C for 60 s, with a final extension step at 72˚C for 10 min. Restriction analysis was performed using the Mspa1I enzyme (New England BioLabs Ltd.). Digestion was performed at 37˚C overnight (1 unit Mspa1I and 10 μl PCR products). The genotypes were then assessed by PAA gel electrophoresis. The fragments were as follows: 183 bp fragment in AA homozygous individuals, three fragments 183bp, 108bp and 75bp in AG heterozygous individuals and 108bp and 75bp fragments in GG homozygous individuals.

The region of the TNF-R2 gene containing the +676 position was amplified using the primers (0.25μM) forward: 5'- TTC TGG AGT TGG CTG CGT GT-3'; reverse: 5'- ACT CTC CTA TCC TGC CTG CT-3'. A 242 bp PCR product was generated using the following amplification conditions: 94˚C for 3 min, followed by 35 cycles at 94˚C for 60 s, 58˚C for 60 s and 72˚C for 60 s, with a final extension step at 72˚C for 10 min. Restriction analysis was performed using Hin1II restriction enzyme (MBI Fermentas, Lithuania). Digestion was performed at 37˚C overnight (1 unit Hin1II and 10 μl PCR products). The genotypes were then assessed by PAA gel electrophoresis. The fragments were as follows: 134 bp and 108 bp fragments in TT homozygous individuals, three fragments 242bp, 134bp and 108bp in TG heterozygous individuals and a 242 bp fragment in GG homozygous individuals.

For all three polymorphisms, genotypes were confirmed by randomly re-genotyping 10% of samples. There were no discrepancies between genotypes determined in duplicate.

### *Statistical analysis*

The Chi square test and Fisher exact test were used to determine possible differences in the genotype and allele frequencies. The association of all three gene variants with risk of disease was examined by unconditional logistic regression analysis to calculate odds ratios (OR) and their 95% confidence intervals (CI). P values of $<0.05$ were considered statistically significant. The expected frequency of variants in controls was analyzed by the Hardy-Weinberg equilibrium test. Calculations were performed using SPSS 10.0 statistical software (SPSS Inc, Chicago, IL, USA).

## RESULTS

No statistically significant difference in genotype and allele distribution between patients and controls could be observed for TNF-alpha and TNF-R1 polymorphisms. However, a difference in genotype and allele frequencies was found between patients and controls for the TNF-R2 polymorphism. Interestingly, for both OSCC and BCC, the SNP at position +676 of the TNF-R2 gene represented a risk factor for tumor development. The observed genotype and allele frequency distribution and risk estimates for polymorphisms in TNF-alpha, TNF-R1 and TNF-R2 genes are given in Tables 1 (OSCC) and 2 (BCC).

## DISCUSSION

There are only a few reports on the possible association of polymorphisms in inflammatory cytokine genes with head and neck cancer, and they mostly concern polymorphisms in interleukin genes. Polymorphism analyses of tumor necrosis factor and/or corresponding receptor genes (TNF-R1 and TNF-R2) are relatively scarce and mainly involve Asian populations (Liu et al., 2005; Gupta et al., 2008; Yang et al., 2011).

Though TNF-alpha gene polymorphisms have been widely studied in various non-malignant and malignant diseases, we found only two studies recently published dealing with TNF-alpha SNP at position -308 as the susceptibility factor for HNSCC development (Correa et al., 2011; Yapijakis et al., 2009). Correa et al. (2011) demonstrated that the -308 allele A and genotype AA in HNSCC individu-

**Table 1**. Genotype and allele frequencies and logistic regression analysis data for the TNF-alpha (-308G>A), TNF-R1 (36A>G) and TNF-R2 (676T>G) polymorphisms in OSCC

| | OSCC (n=50) | control (n=60) | OR | 95% CI | *P* |
|---|---|---|---|---|---|
| | | TNF-alpha (-308G>A) | | | |
| G/G | 35 (69%) | 39 (65%) | 1.00 | Reference | |
| G/A | 14 (29%) | 21 (35%) | 0.74 | 0.33-1.68 | 0.306 |
| A/A | 1 (2%) | 0 (0%) | | | |
| G/A+A/A | 15 (31%) | 21 (35%) | 0.80 | 0.36-1.78 | 0.363 |
| G | 0.84 | 0.83 | 1.00 | Reference | |
| A | 0.16 | 0.17 | 0.93 | 0.44-1.96 | 0.500 |
| TNF-R1 (36A>G) | | | | | |
| A/A | 14 (29%) | 21 (35%) | 1.00 | Reference | |
| A/G | 35 (69%) | 31 (52%) | 1.69 | 0.74-3.89 | 0.150 |
| G/G | 1 (2%) | 8 (13%) | 0.19 | 0.02-1.67 | 0.105 |
| A/G+G/G | 36 (71%) | 39 (55%) | 1.38 | 0.6120-R1 | 0.282 |
| A | 0.64 | 0.61 | 1.00 | Reference | |
| G | 0.36 | 0.39 | 0.88 | 0.50-1.56 | 0.385 |
| TNF-R2 (676T>G) | | | | | |
| T/T | 16 (32%) | 32 (53%) | 1.00 | Reference | |
| T/G | 33 (65%) | 25 (42%) | 2.64 | 1.19-5.84 | 0.013* |
| G/G | 1 (3%) | 3 (5%) | 0.67 | 0.06-6.93 | 0.604 |
| T/G+G/G | 34 (68%) | 28 (47%) | 2.43 | 1.11-5.30 | 0.020* |
| T | 0.65 | 0.74 | 1.00 | Reference | |
| G | 0.35 | 0.26 | 1.53 | 0.84-2.81 | 0.110 |

OR – odds ratio; CI – confidence interval

als might contribute to the higher clinical aggressiveness of malignant disease in the Brazilian population. Similarly, Yapijakis et al. (2009) showed a strong association of TNF-alpha high expression alleles with an increased risk of oral cancer. Though TNF-alpha (-308G>A) is a functional polymorphism that influences TNF-alpha gene transcription and thus the plasma levels of TNF-alpha, in the present study no difference in allele and genotype distribution could be established between cancer patients and controls, excluding this SNP as a potential risk factor for OSCCs in the Serbian population. Since several lines of evidence suggest that an increased expression of TNF-alpha, upregulated by UV exposure, may contribute to tumor escape from the immune response, it seemed reasonable to expect some influence of this SNP on BCC development, as found by Skov et al. (2003). However, our study did not establish any difference in genotype or allele frequencies between BCCs and controls.

The majority of TNF-alpha biological effects are initiated through TNF-R1 (TNFRSF1A), and though the SNP (36G>A) causes only a silent mutation (pro12pro), it may influence mRNA splicing and its cellular localization, resulting in different receptor

**Table 2.** Genotype and allele frequencies and logistic regression analysis data for the TNF-alpha (-308G>A), TNF-R1 (36A>G) and TNF-R2 (676T>G) polymorphisms in BCC

| | BCC (n=50) | control (n=60) | OR | 95% CI | *P* |
|---|---|---|---|---|---|
| | | TNF-alpha (-308G>A) | | | |
| G/G | 29 (58%) | 39 (65%) | 1.00 | Reference | |
| G/A | 21 (42%) | 21 (35%) | 1.34 | 0.62-2.91 | 0.289 |
| A/A | 0 (0%) | 0 (0%) | | | |
| G/A+A/A | 21 (42%) | 21 (35%) | 1.34 | 0.62-2.91 | 0.289 |
| G | 0.79 | 0.83 | 1.00 | Reference | |
| A | 0.21 | 0.17 | 1.30 | 0.64-2.64 | 0.295 |
| TNF-R1 (36A>G) | | | | | |
| A/A | 19 (38%) | 21 (35%) | 1.00 | Reference | |
| A/G | 29 (58%) | 31 (52%) | 1.03 | 0.46-2.30 | 0.549 |
| G/G | 2 (4%) | 8 (13%) | 0.28 | 0.05-1.47 | 0.110 |
| A/G+G/G | 31 (62%) | 39 (56%) | 0.88 | 0.40-1.92 | 0.450 |
| A | 0.67 | 0.61 | 1.00 | Reference | |
| G | 0.33 | 0.39 | 0.77 | 0.43-1.37 | 0.231 |
| TNF-R2 (676T>G) | | | | | |
| T/T | 10 (20%) | 32 (53%) | 1.00 | Reference | |
| T/G | 38 (76%) | 25 (42%) | 4.86 | 2.04-11.62 | <0.001* |
| G/G | 2 (4%) | 3 (5%) | 2.13 | 0.31-14.62 | 0.379 |
| T/G+G/G | 40 (80%) | 28 (47%) | 4.57 | 1.94-10.79 | <0.001* |
| T | 0.58 | 0.74 | 1.00 | Reference | |
| G | 0.42 | 0.26 | 2.06 | 1.13-3.75 | 0.012* |

OR – odds ratio; CI – confidence interval

function. One study showed the interaction of this polymorphism with gastroesophageal reflux disease that influences the risk of esophageal carcinoma (Wu et al., 2011). In our study, no association between a given genotype at position +36 and head and neck cancer risk could be established.

Binding at TNF-R2 (TNFRSF1B) induces apoptosis among CD8+ cells, stimulates the proliferation of hematopoietic cells, and potentiates the effects of TNF at TNF-R1 (Zheng et al., 1995; MacEwan, 2002; Tartaglia et al., 1993). Exon 6 codes for a part of the TNF-R2 transmembrane domain and the site of proteolytic cleavage, resulting in the generation of soluble TNF-R2 (sTNFRII) (Zheng et al., 1995). The polymorphism (676T>G) in exon 6 of TNF-R2 causes the replacement of methionine with arginine at position 196 (M196R) and may potentially modulate the biological effects of TNF since it is located within one of four cysteine-rich domains (CRD) that are important for optimal TNF binding (Morita et al., 2001). In the present study, a significant difference was found in the distribution of allele and genotype frequencies between cases and controls. The as-

sociation between TNF-R2 (676T>G) heterozygous genotype and both OSCC and BCC was statistically significant (OR 2.52; CI 1.13-5.61, p=0.018 and OR 4.67: CI 1.92-11.33, p<0.001, respectively). Carriers of the G allele have an increased risk of developing both oral and skin cancers. Previously, one study showed an association between this SNP and breast cancer onset (Mestiri et al., 2005) and another found association between the SNP and survival in lung cancer (Guan et al., 2011).

Our results suggest that the TNF-R2 gene contributes to a susceptibility to head and neck tumors, and that its aberrant expression or function could be involved in the development of those tumors. To the best of our knowledge, this is the first and unique study to deal with TNF-R2 (676T>G) polymorphism in head and neck malignancies and which establishes the importance of this SNP as a risk factor modulator for two types of tumors with remarkably different biology, OSCCs and BCCs.

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