

## Association of Polymorphisms in *Tumor Necrosis Factor Alpha* and *Beta* Genes with Increased Risk for Oral Cancer

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**Abstract.** *Background:* In light of the recently found contribution of inflammation-related factors to oral oncogenesis, the possible correlation of tumor necrosis factor alpha and beta genes (*TNF- $\alpha$*  and *TNF- $\beta$* ) with risk of oral cancer was investigated. *Materials and Methods:* DNA samples of 160 German and Greek patients with oral squamous cell carcinoma and 153 healthy controls of equivalent age, gender and ethnicity were studied. The functional polymorphisms *TNF- $\alpha$*  (-308 G/A) and *TNF- $\beta$*  (252 G/A), which affect gene expression, were investigated by restriction fragment length polymorphism analysis. *Results:* The frequencies of high expression A2 (-308A) *TNF- $\alpha$*  allele and high expression B1 (252G) *TNF- $\beta$*  allele were significantly increased in cancer patients compared to controls (respectively: 62.2% versus 14.7%,  $p < 0.0001$ ; OR 8.65, 95% CI 5.74-13.04 and 66.9% versus 15.7%,  $p < 0.0001$ ; OR 10.92, 95% CI 7.4-16.2). Three combined *TNF- $\alpha$* /*TNF- $\beta$*  genotypes (A2A2/B1B1, A1A2/B1B2, A1A2/B1B1) were over-represented in cancer patients ( $p < 0.001$ ). No significant differences in allele frequencies were detected among most subgroups of patients divided in regard to cancer stage, family history for cancer or thrombosis, smoking or heavy alcohol consumption habits. *Conclusion:* This study showed a strong association of *TNF- $\alpha$*  and *TNF- $\beta$*  high expression alleles with increased risk of oral cancer. These findings are in accordance with

previously observed high *TNF- $\alpha$*  levels in serum of patients with oral cancer in comparison to healthy controls.

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity (1). Many environmental factors (smoking and alcohol consumption) and genetic factors (oncogenes and suppressor genes) are implicated in the development of oral cancer (1). Several factors related to angiogenesis, inflammation and thrombosis have also been associated with oral oncogenesis (1-2). Such factors previously implicated in cancer, inflammation and thrombotic events are tumor necrosis factor alpha (*TNF- $\alpha$* ) and beta (*TNF- $\beta$* ), which are respectively encoded by *TNF- $\alpha$*  and *TNF- $\beta$*  genes (3).

*TNF- $\alpha$*  is a proinflammatory multifunctional cytokine produced by macrophages (3). It plays an important role in the regulation of immune response since its increase after traumatic injury generates a cytokine cascade resulting in activation, proliferation and hypertrophy of mononuclear and phagocytic cells (4). *TNF- $\alpha$*  has been implicated in the pathogenesis and progression of various malignancies (5). The biological activities of *TNF- $\alpha$*  and the fact that its gene is located within the major histocompatibility complex have suggested that polymorphisms in this locus may be associated with autoimmune, infectious and neoplastic disorders (5).

A G to A transition polymorphism at -308 position of the *TNF- $\alpha$*  gene is important for its expression, since it is situated within the binding site of the AP-2 repressive transcription factor (6). The less common allele A2 (-308A) results in loss of AP-2 binding and, therefore, in increased *TNF- $\alpha$*  gene expression when other repressors are not present (6). The A2 allele frequency ranges between 10-23% in Europeans, 8-10% in South Americans and 2-9% in East Asians (7-9). The presence of the A2 allele has been associated with some malignancies (such as breast and gastric cancer) and possibly with thrombosis (10, 11). Interestingly, other microsatellite polymorphisms located in the *TNF- $\alpha$*  locus have also been associated with an increased

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risk of gastric and colorectal cancer (12). Increased serum levels of TNF- $\alpha$  protein have been observed in European patients with oral cancer, but no association of the high expression A2 allele with increased risk of this malignancy has been found in Taiwanese patients (13, 14).

TNF- $\beta$  is a proinflammatory cytokine produced by lymphocytes and is structurally related to TNF- $\alpha$  (4). The two factors have 30% amino acid sequence identity, are recognized by the same widely distributed cellular receptors and, therefore, share many of their functions (4). The *TNF- $\beta$*  gene is adjacent to the *TNF- $\alpha$*  gene within a 7 kb locus in the major histocompatibility complex (4). A G/A polymorphism located at position 252 within the first intron of the *TNF- $\beta$*  gene affects expression of both genes and concentration of TNF- $\alpha$  and TNF- $\beta$  proteins in plasma (15, 16). The less common allele B1 (252G) has been correlated with a higher TNF- $\beta$  expression both at the mRNA and the protein level (15). On the other hand, the common allele B2 (252A) is associated with increased *TNF- $\alpha$*  gene expression, since it is located within a phorbol ester-responsive DNA element with high affinity for the AP-1, jun and c-fos heterodimer transcription factor family (15). The *TNF- $\beta$*  (G252A) polymorphism has been associated with a risk of development of breast, esophageal, gastric and colorectal cancer (10-12). The reported allele frequency of the high expression B1 homozygotes is about 16% in Europeans and 13% in Asians (10, 17). Interestingly, when the combined *TNF- $\alpha$*  and *TNF- $\beta$*  polymorphisms were examined together in patients with breast or esophageal cancer, certain genotypes were found to be significantly overrepresented and were associated with an increased risk of cancer development (10, 18).

Since the afore mentioned *TNF- $\alpha$*  and *TNF- $\beta$*  polymorphisms may alter concentration of these factors in plasma, and this has already been correlated with cancer, in this study the possible association of these two polymorphisms with the risk of oral cancer in Europeans has been examined.

## Materials and Methods

**Patients.** The subjects under study included 313 Greek and German individuals, consisting of 160 patients with oral cancer and 153 healthy blood donors (controls) of similar age, ethnicity and gender. The patients were mostly men (N=128) and their age ranged between 40-84 years (mean 58.5 $\pm$ 10.1 years). The gender ratio (N=115 men) and the age of the controls (range 38-83 years; mean 56.1 $\pm$ 12.1 years) were comparable to those of the patients.

Patients who had developed OSCC and had been operated recently or up to a decade ago were included in this study. In addition to clinical presentation, biopsy with pathological diagnosis of tumor stages I-IV and a family history regarding cancer and thrombophilia were available. Fifty-eight patients (36.3%) had one or two first-degree relatives with any type of cancer and their age range (mean=58.6 years) did not differ significantly from the whole group of patients. Furthermore, thirty-one patients (19.4%) had one or two first degree relatives with idiopathic thrombosis and an earlier age range (mean=58 years), but again with no statistical difference compared to

the whole group. Fifteen patients (9.4%) had a positive family history for both cancer and thrombophilia (mean age=56.3 years).

Patients and controls were informed about the possible results of the study and willingly donated blood samples.

**DNA isolation and genotyping.** DNA was isolated with the use of a NucleoSpin™ kit (Macherey-Nagel GmbH & Co, Düren, Germany). Genotyping of the two polymorphisms was performed by restriction fragment length polymorphism typing. It involved a combination of PCR amplification and digestion with restriction endonuclease *Nco I*, followed by agarose gel electrophoretic analysis. The PCR amplification consisted of an initial denaturation step at 95°C, followed by 35 cycles of 94°C for 50 s, 63°C (for *TNF- $\alpha$* ) or 68°C (for *TNF- $\beta$* ) for 1 min and 72°C for 55 s, as well as a final step at 72°C for 4 min. For *TNF- $\alpha$*  -308G/A, the primers 5'-AGGCAATAGGTTTGGAGGGCCAT-3' and 5'-TCCTCCCTGCTCCGATTCCG-3' generated a 107 bp PCR product (19). Restriction enzyme digestion with *Nco I* resulted in two fragments (87 and 20 bp), if the A1 allele was present and one intact fragment of 107 bp if the A2 allele was present. For *TNF- $\beta$*  252G/A, the primers 5'-CCGTGCTTCGTGGTTTGGACT-3' and 5'-AGAGGG GTGGATG CTTGGGTTTC-3' generated a 782 bp PCR product (18, 19). Digestion with *Nco I* resulted in two fragments of 586 and 196 bp if the B1 allele was present and a single 782 bp fragment if the B2 allele was present. Some DNA samples were investigated twice for verification of genotyping results.

**Statistical analyses.** The statistical analyses were performed using SAS® software (version 9.0; SAS Worldwide Headquarters SAS Institute Inc., Cary, NC, USA). The frequencies of alleles and genotypes of the whole group or subgroups of patients were compared to the respective frequencies of the control group using the Fisher's exact test (two tailed *p*-values), while all genotype distributions were checked for Hardy-Weinberg equilibrium estimates. The age criterion for the adjustment of odds ratios was set at 60 years. Similar frequency distributions regarding age are found in the respective genotypes between controls and patients. The Mantel-Haenszel method was used for the calculation of all odds ratios with a 95% confidence interval (CI). A *p*-value less than 0.05 was considered statistically significant.

## Results

The prevalence of TNF- $\alpha$  and TNF- $\beta$  polymorphic genotypes of healthy controls (representing the general population) and patients with oral cancer are shown in Tables I-IV. All genotype distributions were as expected in Hardy-Weinberg equilibrium in the control group, as well as in the whole group and subgroups of patients. The data for the Greek and German controls were analyzed together, since there were no significant differences of genotype and allele frequencies among the two studied populations.

In the control group, the observed high expression A2 allele frequency that confers *TNF- $\alpha$*  high expression was 14.7%, and the A2 carrier frequency was 20.9% (Table I). In the patient group, the detected allele and carrier frequencies of the A2 allele were significantly different from those of the control group (62.2% versus 14.7%, and 77.5% versus 20.9%, *p*<0.0001, respectively, Table I). The relative risk (odds ratio, OR) for oral cancer of A2 allele carriers in comparison to A1/A1

Table I. Prevalence of *TNF- $\alpha$*  A1/A2 and *TNF- $\beta$*  B1/B2 polymorphisms in controls and patients with oral cancer (total group). The total group of controls and patients are presented.

| Genotype<br>TNF- $\alpha$                            | Controls    |            | Patients |                   | Genotype<br>TNF- $\beta$                             | Controls    |            | Patients |                    |
|--|-------------|------------|----------|-------------------|--|-------------|------------|----------|--------------------|
|  | n (%)       | n (%)      | p        | OR (CI)           |  | n (%)       | n (%)      | p        | OR (CI)            |
| (A2/A2)  | 13 (8.5%)   | 75 (46.9%) | <0.0001  | 17.51 (8.6-35.6)  | (B1/B1)  | 3 (2.0%)    | 82 (51.3%) | <0.0001  | 40.83 (13.8-120.8) |
| (A1/A1)  | 121 (79.1%) | 36 (22.5%) |          | 1 (referent)      | (B2/B2)  | 108 (70.6%) | 28 (17.5%) |          | 1 (referent)       |
| (A1/A2)  | 19 (12.4%)  | 49 (30.6%) | <0.0001  | 8.33 (4.04-17.15) | (B1/B2)  | 42 (27.5%)  | 50 (31.3%) | <0.0001  | 5.09 (2.69-9.64)   |
| Total  | 153 (100%)  | 160 (100%) |          |                   | Total  | 153 (100%)  | 160 (100%) |          |                    |
| Prevalence<br>of A2 allele                           |             |            |          |                   | Prevalence<br>of B1 allele                           |             |            |          |                    |
| High expression<br>A2 allele frequency               | 14.7%       | 62.2%      | <0.0001  | 8.65 (5.74-13.04) | High expression<br>B1 allele frequency               | 15.7%       | 66.9%      | <0.0001  | 10.92 (7.4-16.2)   |
| Carrier frequency<br>of high expression<br>A2 allele | 20.9%       | 77.5%      | <0.0001  | 12.44 (6.9-22.45) | Carrier frequency<br>of high expression<br>B1 allele | 29.4%       | 82.5%      | <0.0001  | 12.3 (7-21.6)      |

Fischer's *p*-values is for genotype comparisons; odds ratios (OR) are age-adjusted. CI: 95% confidence interval.

Table II. Prevalence of *TNF- $\alpha$*  A1/A2 and *TNF- $\beta$*  B1/B2 polymorphisms in the subgroups of patients in regard to alcohol (no abuse versus abuse) or smoking habits (no abuse versus abuse).

| Genotype<br>TNF- $\alpha$                                  | Tobacco abuse    |              |         |                    | Alcohol abuse    |               |         |                   | Genotype<br>TNF- $\beta$                                   | Tobacco abuse    |              |         |                    | Alcohol abuse    |               |         |                   |
|--|------------------|--------------|---------|--------------------|------------------|---------------|---------|-------------------|--|------------------|--------------|---------|--------------------|------------------|---------------|---------|-------------------|
|  | Patients<br>with |              | without |                    | Patients<br>with |               | without |                   |  | Patients<br>with |              | without |                    | Patients<br>with |               | without |                   |
|  | n (%)            | n (%)        | p       | OR (CI)            | n (%)            | n (%)         | p       | OR (CI)           |  | n (%)            | n (%)        | p       | OR (CI)            | n (%)            | n (%)         | p       | OR (CI)           |
| (A2/A2)  | 73<br>(48%)      | 2<br>(22.2%) | N.S.    | 2.56<br>(0.3-23.0) | 30<br>(57.7%)    | 45<br>(41.3%) | N.S.    | 1.32<br>(0.6-3.2) | (B1/B1)  | 80<br>(52.6%)    | 2<br>(25.0%) | N.S.    | 3.16<br>(0.4-28.5) | 32<br>(61.5%)    | 50<br>(46.3%) | N.S.    | 0.90<br>(0.3-2.4) |
| (A1/A1)  | 34<br>(22.4%)    | 2<br>(22.2%) |         | 1<br>(referent)    | 12<br>(23.1%)    | 24<br>(22.0%) |         | 1<br>(referent)   | (B2/B2)  | 26<br>(17.1%)    | 2<br>(25.0%) |         | 1<br>(referent)    | 10<br>(19.2%)    | 18<br>(16.7%) |         | 1<br>(referent)   |
| (A1/A2)  | 45<br>(29.6%)    | 5<br>(55.6%) | N.S.    | 0.70<br>(0.1-3.5)  | 10<br>(19.2%)    | 40<br>(36.7%) | N.S.    | 0.57<br>(0.2-1.7) | (B1/B2)  | 46<br>(30.3%)    | 4<br>(50.0%) | N.S.    | 0.66<br>(0.1-3.5)  | 10<br>(19.2%)    | 40<br>(37.0%) | N.S.    | 0.48<br>(0.2-1.6) |
| Total  | 152              | 9            |         |                    | 52               | 109           |         |                   | Total  | 152              | 8            |         |                    | 52               | 108           |         |                   |
| Prevalence<br>of A2<br>allele                              |                  |              |         |                    |                  |               |         |                   | Prevalence<br>of B1<br>allele                              |                  |              |         |                    |                  |               |         |                   |
| High<br>expression<br>A2 allele<br>frequency               | 62.8%            | 50.0%        | N.S.    | 2.15<br>(0.7-6.4)  | 67.3%            | 59.6%         | N.S.    | 1.34<br>(0.8-2.2) | High<br>expression<br>B1 allele<br>frequency               | 67.8%            | 50.0%        | N.S.    | 2.85<br>(0.9-9.3)  | 71.2%            | 64.8%         | N.S.    | 1.22<br>(0.7-2.1) |
| Carrier<br>frequency<br>of high<br>expression<br>A2 allele | 77.6%            | 77.8%        | N.S.    | 1.48<br>(0.3-7.0)  | 76.9%            | 78.0%         | N.S.    | 0.91<br>(0.4-2.1) | Carrier<br>frequency<br>of high<br>expression<br>B1 allele | 82.9%            | 75.0%        | N.S.    | 1.97<br>(0.4-9.9)  | 80.8%            | 83.3%         | N.S.    | 0.69<br>(0.3-1.8) |

Fischer's *p*-values are for genotype comparisons; odds ratios (OR) are age-adjusted. CI: 95% confidence interval. N.S.: not significant.

Table III. Prevalence of *TNF-α* A1/A2 polymorphism in the subgroups of patients in regard to tumor stages (early versus late), family history of cancer (negative versus positive) or thrombophilia (negative versus positive).

| Genotype<br><i>TNF-α</i>   | Patients with cancer |                  |          |                   | Family history of cancer |                     |          |                    | Family history of thrombophilia |                     |          |                   |
|--|----------------------|------------------|----------|-------------------|--------------------------|---------------------|----------|--------------------|---------------------------------|---------------------|----------|-------------------|
|  | Stages<br>I&II       | Stages<br>III&IV | <i>p</i> | OR(CI)            | Patients<br>with         | Patients<br>without | <i>p</i> | OR(CI)             | Patients<br>with                | Patients<br>without | <i>p</i> | OR(CI)            |
|  | n (%)                | n (%)            |          |                   | n (%)                    | n (%)               |          |                    | n (%)                           | n (%)               |          |                   |
| (A2/A2)  | 44<br>(49.4%)        | 31<br>(43.1%)    | N.S.     | 1.10<br>(0.5-2.7) | 31<br>(52.5%)            | 44<br>(43.1%)       | N.S.     | 3.68<br>(1.3-10.2) | 10<br>(32.3%)                   | 65<br>(50%)         | N.S.     | 1.01<br>(0.3-4.1) |
| (A1/A1)  | 20<br>(22.5%)        | 16<br>(22.2%)    |          | 1<br>(referent)   | 8<br>(13.6%)             | 28<br>(27.5%)       |          | 1<br>(referent)    | 4<br>(12.9%)                    | 32<br>(24.6%)       |          | 1<br>(referent)   |
| (A1/A2)  | 25<br>(28.1%)        | 25<br>(34.7%)    | N.S.     | 1.59<br>(0.6-4.2) | 20<br>(33.9%)            | 30<br>(29.4%)       | N.S.     | 3.80<br>(1.3-11.2) | 17<br>(54.8%)                   | 33<br>(25.4%)       | 0.0211   | 2.57<br>(0.7-9.0) |
| Total  | 89                   | 72               |          |                   | 59                       | 102                 |          |                    | 31                              | 130                 |          |                   |
| Prevalence<br>of A2 allele<br>High<br>expression<br>A2 allele<br>frequency | 63.5%                | 60.4%            | N.S.     | 0.97<br>(0.6-1.6) | 69.5%                    | 57.8%               | 0.0429   | 2.05<br>(1.2-3.4)  | 59.7%                           | 62.7%               | N.S.     | 0.86<br>(0.5-1.6) |
| Carrier<br>frequency<br>of high<br>expression<br>A2 allele                 | 77.5%                | 77.8             | N.S.     | 1.27<br>(0.6-3.0) | 86.4%                    | 72.6%               | N.S.     | 3.68<br>(1.4-9.5)  | 87.1%                           | 75.4%               | N.S.     | 1.43<br>(0.4-4.7) |

Fischer's *p*-values is for genotype comparisons; odds ratios (OR) are age-adjusted. CI: 95% confidence interval. N.S.: not significant.

homozygotes was quite high (OR>8, Table I). Furthermore, homozygotes for the high expression allele (A2/A2) seem to have a double risk of developing oral cancer than A1/A2 heterozygotes (OR 17.51 versus OR 8.33, Table I).

Statistical comparisons of *TNF-α* A2 allele frequency among subgroups of patients in regard to tobacco or alcohol abuse, stage of cancer, as well as family history of cancer or thrombosis are shown in Tables II and III. No significant differences were revealed among those subgroups, except a significant increase of high expression A2 alleles in patients with a positive family history of cancer in comparison to patients without one (*p*=0.0429, Table III). Additionally, although the number of patients with positive family history of thrombosis was limited, A1/A2 heterozygotes with a positive family history of thrombophilia were significantly increased compared to patients without a family history (*p*=0.0211, Table III).

With regards to *TNF-β* genotypes, the observed high expression B1 allele frequency in the control group was 15.7% and the B1 carrier frequency was 29.4% (Table I). In the patients group, the observed allele and carrier frequencies of the high expression B1 allele were significantly increased compared to those of the control group (66.9% versus 15.7%, and 82.5% versus 29.4%, respectively, Table I). B1 allele carriers have at least 5 times greater risk of oral cancer than B2/B2 homozygotes

(OR 5.09, 95% CI 2.69-9.64, Table I). Homozygotes for the high expression allele (B1/B1) seem to have 8 times more risk of developing oral cancer than B1/B2 heterozygotes (OR 40.83 versus OR 5.09, Table I). Furthermore, there was no significant difference in *TNF-β* B1 allele frequency among subgroups of patients with regards to tobacco or alcohol abuse, cancer stage, and family history of cancer or thrombophilia (Tables II and IV).

Finally, the A1A1/B2B2 genotype was used as reference for statistical comparison of the prevalence of combined *TNF-α*/*TNF-β* genotypes, since A1 and B2 are both more common and low expression alleles (Table V). All genotypes, except A1A2/B2B2, were significantly different among patients and controls (Table V). Three genotypes (A2A2/B1B1, A1A2/B1B2, A1A2/B1B1) were over-represented in patients with OSCC, while two other ones (A1A1/B2B2, A1A1/B1B2) were under-represented.

## Discussion

High *TNF-α* and *TNF-β* expression has been associated with malignancy and metastasis (3, 5). Two common functional *TNF-α* and *TNF-β* polymorphisms, which affect gene expression, have been implicated in several types of cancer (10-12). The purpose of this study was to examine the possible

Table IV. Prevalence of *TNF- $\beta$*  B1/B2 polymorphism in the subgroups of patients in regard to tumor stages (early versus late), family history of cancer (negative versus positive) or thrombophilia (negative versus positive).

| Genotype<br><i>TNF-<math>\beta</math></i>                                  | Patients with cancer |                  |          |                   | Family history of cancer |                     |          |                   | Family history of thrombophilia |                     |          |                    |
|--|----------------------|------------------|----------|-------------------|--------------------------|---------------------|----------|-------------------|---------------------------------|---------------------|----------|--------------------|
|  | Stages<br>I&II       | Stages<br>III&IV | <i>p</i> | OR (CI)           | Patients<br>with         | Patients<br>without | <i>p</i> | OR (CI)           | Patients<br>with                | Patients<br>without | <i>p</i> | OR (CI)            |
|  | n (%)                | n (%)            |          |                   | n (%)                    | n (%)               |          |                   | n (%)                           | n (%)               |          |                    |
| (B1/B1)  | 52<br>(59.1%)        | 30<br>(41.7)     | N.S.     | 0.58<br>(0.2-1.4) | 34<br>(58.6%)            | 48<br>(47.1%)       | N.S.     | 1.27<br>(0.5-3.4) | 14<br>(46.7%)                   | 68<br>(52.3%)       | N.S.     | 1.16<br>(0.3-3.9)  |
| (B2/B2)  | 14<br>(15.9%)        | 14<br>(19.4%)    |          | 1<br>(referent)   | 10<br>(17.2%)            | 18<br>(17.7%)       |          | 1<br>(referent)   | 4<br>(13.3%)                    | 24<br>(18.5%)       |          | 1<br>(referent)    |
| (B1/B2)  | 22<br>(25.0%)        | 28<br>(38.9%)    | N.S.     | 0.98<br>(0.4-2.7) | 14<br>(24.1%)            | 36<br>(35.3%)       | N.S.     | 0.46<br>(0.1-1.5) | 12<br>(40.0%)                   | 38<br>(29.2%)       | N.S.     | 1.01<br>(0.3-3.91) |
| Total  | 88                   | 72               |          | 58                | 102                      |                     | 30       | 130               |                                 |                     |          |                    |
| Prevalence<br>of B1 allele<br>High<br>expression<br>B1 allele<br>frequency | 71.6%                | 61.1%            | N.S.     | 0.68<br>(0.4-1.1) | 70.7%                    | 64.7%               | N.S.     | 1.40<br>(0.8-2.4) | 66.7%                           | 66.9%               | N.S.     | 1.02<br>(0.8-1.9)  |
| Carrier<br>frequency<br>of high<br>expression<br>B1 allele                 | 84.1%                | 80.6             | N.S.     | 0.77<br>(0.3-1.8) | 82.8%                    | 82.4%               | N.S.     | 0.98<br>(0.4-2.5) | 86.7%                           | 81.5%               | N.S.     | 1.15<br>(0.4-3.5)  |

Fischer's *p*-values is for genotype comparisons; odds ratios (OR) are age-adjusted. CI: 95% confidence interval. N.S.: not significant.

Table V. Analysis of combined *TNF- $\alpha$ /TNF- $\beta$*  genotypes in controls and patients with oral cancer.

| <i>TNF-<math>\alpha</math>/TNF-<math>\beta</math></i><br>Genotype | Controls<br>(%) | Patients<br>(%) | Total | <i>p</i> -Value | OR           | 95% CI        |
|---|-----------------|-----------------|-------|-----------------|--------------|---------------|
| A1A1/B2B2   | 95 (62.%)       | 18 (11.2%)      | 113   |                 | 1 (referent) |               |
| A1A1/B1B2   | 26 (17%)        | 16 (10%)        | 42    | 0.046           | 3.10         | (1.17-8.16)   |
| A1A1/B1B1   | 0 (0%)          | 2 (1.3)         | 2     | 0.029           | 16.76        | (0.7-384.1)   |
| A1A2/B2B2   | 12 (7.8%)       | 6 (3.7%)        | 18    | N.S.            | 2.26         | (0.65-7.89)   |
| A1A2/B1B2   | 7 (4.6%)        | 28 (17.5%)      | 35    | <0.001          | 14.93        | (5.45-40.89)  |
| A1A2/B1B1   | 0 (0%)          | 15 (9.4%)       | 15    | <0.001          | 61.48        | (7.61-497.02) |
| A2A2/B2B2   | 1 (0.7%)        | 4 (2.5%)        | 5     | 0.042           | 12.28        | (1.69-89.6)   |
| A2A2/B1B2   | 9 (5.9%)        | 6 (3.8%)        | 15    | 0.0361          | 16.76        | (0.73-384.05) |
| A2A2/B1B1   | 3 (2%)          | 65 (40.6%)      | 68    | <0.0001         | 46.64        | (14.74-147.6) |
| Total   | 153             | 160             | 313   |                 |              |               |

contribution of these *TNF- $\alpha$*  and *TNF- $\beta$*  gene polymorphisms in the development of oral cancer. The participants included patients with OSCC and healthy population-based controls of matched ethnicity, age and gender.

Although the total number of studied individuals (N=313) was modest, the results of this study clearly indicated a very strong association of alleles A2 and B1 (resulting in high expression of *TNF- $\alpha$*  and *TNF- $\beta$*  genes, respectively) with oral

oncogenesis. A highly significant increase of carrier and allele frequencies for the high expression A2 and B1 alleles were observed in patients compared to controls. The same highly significant increase in comparison to healthy controls was observed in all subgroups of patients with regards to initial or advanced cancer stage, family history for cancer or thrombosis, smoking or heavy alcohol consumption habits. As a rule, the patient subgroups did not differ significantly among them with

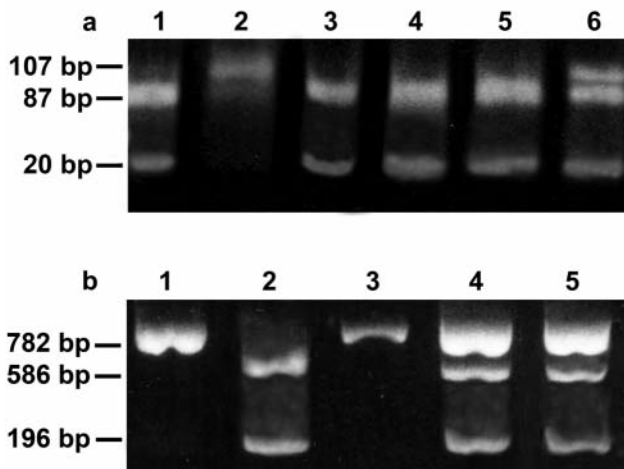


Figure 1. Illustrative example of observed *TNF-α* A1/A2 and *TNF-β* B1/B2 genotypes. a) Agarose gel electrophoresis of *Nco* I-digested PCR products for genotyping the -308 *TNF-α* A1/A2 polymorphism in patients with oral cancer. The A1 allele corresponds to two fragments of 87 and 20 bp, while the A2 allele to a single fragment of 107 bp. 1,3,4,5: A1/A1 homozygotes; 2: A2/A2 homozygote; 6: A1/A2 heterozygote. b) Agarose gel electrophoresis of *Nco* I-digested PCR products for genotyping the 252 *TNF-β* B1/B2 polymorphism in patients with oral cancer. The B1 allele corresponds to two fragments of 586 and 196 bp, while the B2 allele to a single fragment of 782 bp. B2/B2 homozygotes: 1,3; B1/B1 homozygote: 2; B1/B2 heterozygotes: 4,5.

respect to the studied *TNF-α* and *TNF-β* allele frequencies, indicating that these polymorphisms are not correlated to smoking or alcohol abuse, tumor stage, and in most cases with family history.

The only significant increase in high expression A2 alleles was observed in the subgroup of patients with a positive family history for cancer, probably underscoring the promoting effect of this polymorphic allele in other types of malignancies as well. In accordance with this notion, a significant increase of A2 allele homozygotes has been reportedly observed among patients with non-Hodgkin's lymphomas, non-small cell lung cancer, breast cancer, gastric carcinoma and hepatocellular carcinoma (10-12, 20-22).

Most of the previous studies, which attempted to directly investigate the transcriptional effects of the -308G/A polymorphism using transient transfections of human or rodent cell lines, have demonstrated that the A2 allele is a powerful activator of *TNF-α* gene expression *in vitro*, compared to the A1 allele (6, 23-25). Only three out of ten such studies failed to demonstrate any difference in transcription driven by either promoter allele, possibly reflecting differences in vector constructs, cell lines, kinetics or stimuli (6, 23-25). Nevertheless, it seems that the effect of the A2 allele on *TNF-α* gene expression is exerted only in the absence of certain repressors, such as GCF2/LRRFIP1, or in the presence of activators, such as Ets-1 (25). Interestingly, reports have

previously shown the important role of transcription factor Ets-1 in the oral region, both in up-regulating *TNF-α* gene expression and in its involvement in carcinogenesis (26, 27).

On the other hand, the B1 allele of the 252G/A polymorphism has repeatedly been correlated with a higher *TNF-β* gene expression both at the mRNA and the protein level, while the common allele B2 has been associated with increased *TNF-α* gene expression (15). It seems that *TNF-β* *per se* contributes in certain malignancies, since the high expression B1 allele has been detected to be significantly increased in patients with osteosarcoma, as well as breast, colorectal and bladder cancer (11, 12, 28, 29).

In accordance to the present study, increased levels of *TNF-α* have been observed in European patients with oral cancer in comparison to healthy subjects (13). The tumorigenic role of *TNF-α* has emerged only recently. True to its name, high doses of soluble *TNF-α* may cause hemorrhagic necrosis by selective destruction of tumor blood vessels and generation of specific anti-tumor T-cell response (30). Nevertheless, when produced in tumor cells *TNF-α* enhances malignant cell growth, survival, invasion and metastasis because it occurs as a transmembrane molecule, made soluble upon activation of a protease (31-33). Endogenous membrane *TNF-α* can induce angiogenesis by stimulation of vascular endothelial growth factor (VEGF), interleukines IL-6 IL-8 and their receptors, as well as tissue remodeling by induction of matrix metalloproteinases, such as MMP-9 (32, 33). The findings of this study, which suggest a significant role of highly expressed endogenous *TNF-α* in oral oncogenesis, are in accordance to previous studies that associated high gene expression of IL-8 and MMP-9 with an increased risk of early stages of oral cancer (2, 34, 35). Furthermore, it has been previously found that a strong association with oral oncogenesis exists, not with a polymorphic allele resulting in constitutively high *IL-6* gene expression, but with an allele which increases *IL-6* gene transcription only after an inflammatory stimulus (36). Interestingly, the latter study was also in accordance to the present one, since constitutive production of high *IL-6* levels may suppress *TNF-α* gene expression (37). Although less studied, *TNF-β* probably has a similar tumorigenic role as *TNF-α*, since it is known also to be a potent inducer of some factors such as IL-6 (37, 38). In addition, *TNF-α* and *TNF-β* have already been implicated in prothrombotic events through enhanced expression of plasminogen activator inhibitor 1, the high expression of which has also been associated with an increased risk of oral cancer (39).

Two previous studies of the *TNF-α* -308G/A polymorphism in Taiwanese Chinese reported contradictory results among them and with the findings of the present study. One did not find any association with OSCC, while the other detected a significant increase of the low expression A1 allele in patients in comparison to controls (14). This discrepancy might be explained with the diverse ethnic background between the studied populations (European and Chinese).

Alternatively, the findings of this study might be explained with the possible existence of another susceptibility gene adjacent to the *TNF- $\alpha$*  and *TNF- $\beta$*  genes. Hypothetically, a polymorphic allele in such a presently unknown gene might be in linkage disequilibrium with the TNF genetic markers located within the major histocompatibility complex class III region. Previous studies in immune-related diseases have found an association of *TNF- $\alpha$*  A2 allele with HLA-DR17 in Scandinavian patients with sarcoidosis, as well as differences in *TNF- $\beta$*  alleles and linkage to HLA-B8-DR3 among European and Japanese patients with systemic lupus erythematosus (40-42). Interestingly, specific expression of HLA-DR antigens in well-differentiated OSCC has been reportedly associated significantly with unfavorable prognosis (36). The above mentioned differences in the frequency of *TNF- $\alpha$*  alleles associated with oral cancer between Europeans and Chinese support this notion.

The combined analysis of *TNF- $\alpha$*  and *TNF- $\beta$*  genotypes detected an over-representation of genotypes A2A2/B1B1, A1A2/B1B2 and A1A2/B1B1. The fact that B1-containing genotypes were over-represented in patients with OSCC indicates that, in addition to the *TNF- $\alpha$*  gene, *TNF- $\beta$*  also plays an important role in the increasing risk of oral cancer, since B1 elevates *TNF- $\beta$*  production (15). Interestingly, the genotype A1A2/B1B2 has been reportedly associated with a reduced risk of developing esophageal and gastric cancer in Chinese, and an increased risk of myeloma in English Caucasians (10, 38). Again, ethnic differences between Europeans and Chinese might account for these discrepancies.

In conclusion, two common functional polymorphisms in *TNF- $\alpha$*  and *TNF- $\beta$*  genes seem to increase the risk of development of oral cancer in Europeans. The detection of such genetic predisposition may be of considerable importance for safeguarding the life and health status of certain individuals at risk in the general population through early prevention measures.

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