A DNA Polymorphism of Stromal-derived Factor-1 is Associated with Advanced Stages of Oral Cancer

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Abstract. Background: Stromal derived factor-1 (SDF-1), a protein related to angiogenesis and inflammation, has been correlated with the progression of a number of malignancies, but not with oral squamous cell carcinoma. In light of the known contribution to the development of oral cancer of other gene polymorphisms for proteins responsible for angiogenesis, inflammation and thrombosis, this study investigated whether the G801A polymorphism in the SDF-1 gene is associated with this malignancy. Patients and Methods: The G801A polymorphism was examined by restriction fragment length polymorphism analysis in DNA samples from 159 patients with oral squamous cell carcinoma and 101 matched healthy controls. Results: The detected allele frequency of the "high production related" A allele in the control group was 25.3%. There was a slight decrease in allele frequency in patients (18.6%), but it was not statistically significant. The same pattern was observed in subgroups of patients in regard to smoking habits and family history of cancer or thrombosis. Interestingly, in comparison to controls, the A allele frequency was significantly lower in patients with oral squamous cell carcinoma and in patients with advanced cancer stages III and IV (12.5%, p=0.005) and in patients with alcohol abuse (12.5%, p=0.02). Conclusion: The G801A polymorphism of the SDF-1 gene is associated with advanced stages of oral cancer, especially in alcohol abusers.

Oral squamous cell carcinoma is the most frequent histopathological form of oral cancer, characterized by a low survival rate (1). Its development is considered to be a multistep process provoked by other factors, such as alcohol and tobacco abuse, along with gene alterations in oncogenes and tumor suppressor genes (1). Recently, its pathogenesis has also been correlated with polymorphisms in genes of proteins responsible for inflammation, angiogenesis and thrombosis (2-9). This warrants further research on such inherited polymorphisms with the hope that it might lead to better prevention and improvement in the presently low survival rate.

Stromal derived factor-1 (SDF-1), also known as CXCL12, is an alpha chemokine involved both in angiogenesis and tumor growth (10). Its role in physiological and pathological processes is exerted through interactions with its G-protein-coupled receptor CXCR4 (11). SDF-1 was originally found to support the proliferation of B-cell progenitors in the presence of interleukin-7, but it is mainly involved in the formation of new blood vessels by hematopoietic cells through interactions with vascular endothelial growth factor (VEGF) (12). Recent studies have showed that organ-specific expression of VEGF is sufficient to mobilize and recruit hematopoietic cells from the bone marrow to the blood, but retention of the proangiogenic subpopulation of hematopoietic cells in peripheral organs requires SDF-1 (12). Furthermore, it has a crucial role in embryonic development, since experimental deficiency of SDF-1 or its receptor CXCR4 in the developing murine embryo results in early lethal defects involving defective blood vessels and cardiac, gastrointestinal and nervous system malformations (13).

SDF-1 seems to be significant for tumor growth, angiogenesis and metastasis, and contributes to immunosuppressive networks within the tumor microenvironment (10). Increased levels of SDF-1 have been correlated with the progression of a number of malignancies such as prostate cancer, small cell lung carcinoma, pancreatic cancer and glioblastoma, as well as metastasis of breast cancer cells to bone and survival of B-chronic lymphoblastic leukemia
(14-19). SDF-1 is also connected with allergic airway disease and inflammatory diseases, such as idiopathic inflammatory myopathies, and progression of HIV infection, since the CXCR4 receptor is used by the HIV-1 to enter target cells (20-23).

A single nucleotide polymorphism (G to A) exists at position 801 in the 3'-untranslated region of the SDF-1 gene (24). Initially, it was hypothesized that the A allele might serve as a target for cis-acting factors, capable of upregulating the expression of SDF-1 gene (24-26). Subsequently, it was shown that the SDF-1 G801A genotype did not significantly affect the mRNA levels in virus-transformed lymphoblastoid cell lines (27). Nevertheless, the less common A allele of the G801A polymorphism has been found in linkage disequilibrium with haplotypes associated with increased amount of SDF-1 transcripts and higher production of SDF-1 protein (28). Therefore, the mutant A allele is considered to be related with "high production" of SDF-1 compared to the G allele (28). The A allele is carried by 25-30% of Caucasians (29-31). The presence of the G801A polymorphism has been associated with the clinical presentation and the risk of distant tissue infiltration by tumor cells in acute myeloid leukemia and with increased susceptibility of breast and lung cancer in some populations (32-34).

Considering the significance of the chemokine SDF-1 in the angiogenesis and progression of various malignancies, we investigated the association of the SDF-1 G801A polymorphism with risk for oral cancer by studying a cohort of patients with oral cancer and healthy controls representing the general population.

**Patients and Methods**

The individuals under study were 260 Greeks and Germans, recruited by the participating departments. They included 159 patients with squamous cell carcinoma in the oral cavity and 101 healthy blood donors of similar age, ethnicity and gender.

The patients included in this study had developed oral cancer and were operated on recently or up to a decade ago. In addition to clinical presentation, a biopsy with pathological diagnosis of tumor stages I-IV and a family history regarding cancer and thrombophilia were available. Fifty-four patients (34%) had one or two first-degree relatives with some type of cancer and their age, mean 58.9±10.1 years did not differ significantly from the whole group of patients. Furthermore, thirty patients (18.9%) had one or two first-degree relatives with idiopathic thrombosis and an age (44-75 years; mean age 58.5±10.0 years) again not statistically different from the group as a whole. Seventeen patients (8.8%) had a positive family history for both cancer and thrombophilia (48-74 years; mean age 57.2±8.2 years).

Most of the participants in the two groups worked in a low-risk environment (with the exception of one patient and three controls who worked in chemical factories). No data were available on controls regarding their family history or smoking and alcohol consumption habits.

Blood samples were collected from the patients and controls under study after informed consent had been obtained. DNA was isolated from blood with the use of Nucleon™ kits (Amersham, Buckinghamshire, UK). Molecular detection of the G801A polymorphism in the SDF-1 gene was performed by restriction fragment length polymorphism typing. This involved a combination of PCR amplification followed by gel electrophoretic analysis. The PCR conditions consisted of an initial denaturation step at 94°C, followed by 35 cycles of 94°C for 45 s, 62°C for 30 min, and 72°C for 30 s, as well as a final elongation step at 72°C for 5 min. The primers used were: forward: 5'-CAGTCAACCTGGGCAAAGCC-3' and reverse: 5'-AGCTTT GGTCTGTAGAGTCC-3'. The PCR products with the SDF1-3' G allele produced 2 discrete fragments of 99 and 203 bp, whereas those with the SDF1-3' A allele produced 1 fragment of 302 bp (35).

The statistical analyses were performed using SAS® software (version 9.0, SAS Institute Inc, Cary, NC, USA). The frequencies of alleles and genotypes (with the GG genotype as referent) of the whole group or subgroups of patients were compared to the respective frequencies of the control group using Fisher's exact test and age-adjusted odds ratios, while all genotype distributions were checked for compliance with Hardy-Weinberg estimates. The Maentel-Haenzel 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**

Genotypes, allele and carrier frequencies in healthy controls, patients with oral cancer and patients subgroups are shown in Table I. The data for the two tested populations (Greek and German healthy controls) were analyzed together, since there were no significant differences in allele frequencies of the G801A polymorphism among the two populations. The observed genotypes in the control group resulted in a "high production related" allele A frequency of 25.3%, which is similar to other Caucasian populations (29-31), and a carrier frequency of 45.5% (Table I). All G801A genotype distributions were in Hardy-Weinberg equilibrium in the control group, as well as in the whole group and subgroups of patients.

The detected genotypes in the patient group (Table I) resulted in no significant difference of A allele and A allele carrier frequencies compared to the equivalent ones in the control group (18.6% vs. 25.25%, p=0.08 and 34.59% vs. 45.54%, p=0.09, respectively). No significant difference of mutant A allele or A allele carrier frequencies in comparison to controls was also observed in subgroups of patients: a) with early (I,II) stages of cancer (genotypes AA=2, GG=46, GA=34, p=0.71 and p=0.88, respectively, Table I), b) with positive family history of cancer (genotypes AA=2, GG=36, GA=16, p=0.20 and p=0.17, respectively), c) without positive family history of cancer (genotypes AA=2, GG=60, GA=30, p=0.11 and p=0.14, respectively), d) with positive family history of thrombophilia (genotypes AA=2, GG=36, GA=16, p=0.20 and p=0.17, respectively).
AA=0, GG=18, GA=12, p=0.49 and p=0.68, respectively), e) without positive family history of thrombophilia (genotypes AA=4, GG=78, GA=34, p=0.08 and p=0.07, respectively), f) with nicotine abuse (genotypes AA=4, GG=88, GA=44, p=0.11 and p=0.14, respectively), g) without nicotine abuse (genotypes AA=0, GG=8, GA=2, p=0.17 and p=0.18, respectively), and h) without alcohol abuse (genotypes AA=4, GG=60, GA=34, p=0.41 and p=0.39, respectively).

In comparison to controls, significant decrease in both "high production-related" A allele and A allele carrier frequencies were observed in the subgroups of patients with cancer stages III and IV (p=0.005 and p=0.002, respectively, Table I) and alcohol abusers (p=0.02 and p=0.02, respectively, Table I). Even in those subgroups of patients, the relative risk (OR) for oral cancer was rather small (Table I).

### Discussion

SDF-1 is a homeostatic chemokine that plays an important role in angiogenesis, organization of the immune system and tumor development by signaling through its exclusive receptor, CXCR4 (11). Increased frequency of the "high production-related" A allele in the SDF-1 G801A polymorphism has been associated with increased susceptibility to lung and breast cancer (32, 33).

The purpose of this study was to investigate whether the G801A polymorphism is related to risk for oral cancer in Europeans. Despite the modest number of studied individuals, no major association of the studied SDF-1 polymorphism with risk for oral cancer was revealed. The A allele and carrier frequencies in the whole group and in almost every subgroup of patients were not significantly different from those of the respective frequencies of the control group. Nevertheless, both these frequencies were significantly lower in patients with cancer stages III and IV and in patients with alcohol abuse, without affecting much the relative risk for oral cancer.

The interpretation of these unexpected findings may illuminate the role of SDF-1 in oral carcinogenesis. It is known that SDF-1 and its receptor CXCR4 exert their effects in neoplasias mainly by regulating crosstalk between tumor cells and their microenvironment (36). Within the tumor microenvironment, a large proportion of the non-neoplastic cells constitutively secrete SDF-1, which in turn attracts tumor cells, acting through the CXCR4 receptor expressed by them (36). Therefore, CXCR4 is essential for metastatic spread to organs where SDF-1 is expressed, allowing tumor cells to access several cellular niches, including hemopoietic marrow. Furthermore, SDF-1 not only stimulates survival and growth of neoplastic cells in a paracrine fashion, but it also

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls No. (%)</th>
<th>Patients with cancer stages I &amp; II No. (%)</th>
<th>Patients with cancer stages III &amp; IV No. (%)</th>
<th>Patients with alcohol abuse No. (%)</th>
</tr>
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<tbody>
<tr>
<td>Mutant:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5 (5%)</td>
<td>4 (2.5%)</td>
<td>2 (2.4%)</td>
<td>0 (0.00%)</td>
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<tr>
<td>Normal:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GG</td>
<td>55 (54.5%)</td>
<td>104 (65.4%)</td>
<td>1 (56.1%)</td>
<td>1 (75.00%)</td>
</tr>
<tr>
<td>Carrier:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GA</td>
<td>41 (40.6%)</td>
<td>51 (32.01%)</td>
<td>34 (41.5%)</td>
<td>12 (25.00%)</td>
</tr>
<tr>
<td>Total</td>
<td>101 (100%)</td>
<td>159 (100%)</td>
<td>82 (100%)</td>
<td>48 (100%)</td>
</tr>
</tbody>
</table>

Prevalence of T allele
A allele frequency 25.3% 18.6% 0.08 0.65 23.2% 0.71 0.90 12.5% 0.005 0.45 12.5% 0.02 0.46
Carrier frequency of A allele 45.5% 34.6% 0.09 0.60 43.9% 0.88 0.94 21.9% 0.002 0.35 25.0% 0.02 0.42

The significant p-values are illustrated in bold characters.
promotes tumor angiogenesis by attracting endothelial cells to the tumor microenvironment (36). Over the past 5 years, several neoplasias have been described to involve CXCR4 activation by SDF-1, including breast cancer, small cell lung carcinoma, pancreatic cancer, prostate cancer, glioblastoma and others (14-19). Accordingly, the findings of this study revealed no statistical difference in "high production-related" A allele frequency between patients with early cancer stages and healthy controls, while the A allele frequency was significantly lower in patients with advanced cancer stages. Interestingly, it would seem as if the "high production-related" A allele might play a prophylactic role in the advancement of oral oncogenesis.

A possible explanation of the discrepancy might involve the rich vasculature of the oral mucosa (9, 37). In the oral microenvironment, even when the "low production-related" G allele is present, sufficient SDF-1 levels may be available stimulating survival, growth and metastasis of neoplastic cells, acting through tumor-expressed CXCR4 receptor. In advanced cancer stages, low levels of SDF-1 may have multiple effects by retaining the proangiogenic subpopulation of hematopoietic cells, as well as by interacting with released matrix metalloproteinase-9 (MMP-9), an important matrix degradation enzyme, previously associated with risk for oral cancer (12). The role of angiogenesis itself in oral cancer is in dispute, underscoring the difficulty of evaluating tumor-associated neovascularization in the oral mucosa due to its rich vasculature (38). Interestingly, low levels of another proangiogenic factor, VEGF, were found to be associated with increased risk for early stages of oral cancer (9).

In regard to the observed significant decrease of the "high production-related" A allele in oral cancer patients with heavy alcohol consumption, a plausible explanation seems to be in accordance with the known effects of alcohol on SDF-1/CXCR4 signaling. The latter acts through induction of CXCR4 internalization (39), but alcohol is known to increase intracellular cAMP, which in turn significantly reduces CXCR4 receptor internalization and increases the re-externalization rate (40). Therefore, in the presence of alcohol, the putative prophylactic effect of the A allele of SDF-1 is minimized.

In conclusion, the investigated SDF-1 polymorphism revealed an association with advanced stages of oral cancer in a subset of the general population, especially heavy drinkers. It is of great importance to perform further genetic association studies, which may ultimately result in symptomatic testing and preventive measures safeguarding the health status and lives of certain at risk individuals in the general population.

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