High Gene Expression of Matrix Metalloproteinase-7 is Associated with Early Stages of Oral Cancer

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Abstract. *Background: In the light of the recently found contribution of matrix metalloproteinases (MMPs) to oral oncogenesis, the correlation of MMP-7 with risk for oral cancer was investigated. Materials and Methods: The MMP-7 -181A/G polymorphism in 159 German and Greek patients with oral squamous cell carcinoma and 120 healthy controls of equivalent gender and ethnicity was studied. Results: The detected carrier frequency of the high expression G allele was significantly higher in patients compared to controls (74.8% versus 61.7%, p=0.0257). This significant difference was more pronounced in patients with early stages of cancer and absent in those with advanced stages. A/G heterozygotes have a double relative risk (OR 2.07, 95%, CI 1.17-3.67) of developing early stages of oral cancer than low expression A/A homozygotes. Conclusion: MMP-7 gene expression is associated with increased risk only for early stages of oral cancer, possibly due to the inhibitory effect of MMP-7 in angiogenesis.*

Oral squamous cell carcinoma (OSCC) is the sixth most common and fatal human malignancy worldwide (1). Recently, in addition to well-known factors, such as smoking, alcohol abuse and genetic alterations in oncogenes and tumor suppressor genes, factors involved in thrombosis and angiogenesis have also been implicated in increased risk for tumor development in the oral cavity (2-10). One such factor is the membrane-type matrix metalloproteinase-7 (MMP-7), which has been strongly associated with angiogenesis, tumor development, invasion, metastasis and thrombosis (11-18).

MMP-7, also known as matrilysin, is a member of the MMP family of proteases which degrade the extracellular matrix and, therefore, facilitate invasion of the surrounding connective tissue by neoplastic cells (11-13). MMP-7 is epithelium-specific and has a broad activity for several substrates, including elastin, proteoglycans, fibronectin and collagen type IV (17, 19). MMP-7 plays a pivotal role in inflammatory diseases and malignant invasion by tissue remodeling (17). Increased expression of MMP-7 has been observed in many types of cancer including oral, esophageal, gastric, endometrial, colorectal and pancreatic ductal carcinoma (13, 20-26). Interestingly, MMP-7 has been associated only with early stages of colorectal carcinogenesis, possibly because it generates angiostatin from plasminogen (27, 28). Angiostatin is known to be a strong inhibitor of angiogenesis and, therefore, limits tumor growth and invasion (28).

A single nucleotide polymorphism (-181A/G) in the promoter region of the *MMP-7* gene has been shown to influence gene expression *in vitro* (29). The presence of the G allele is associated with a 2- to 3-fold higher transcriptional activity than with the A allele, because it reportedly facilitates the binding of nuclear activating proteins in the promoter of the *MMP-7* gene (29). The G allele is common (about 40-43%) in Caucasians and rare (2.5-5%) in East Asians (29-35). Several studies reported a significant increase of the high expression G allele in patients with various types of cancer, incuding esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma, adult astrocytoma, non-small cell lung carcinoma, colorectal

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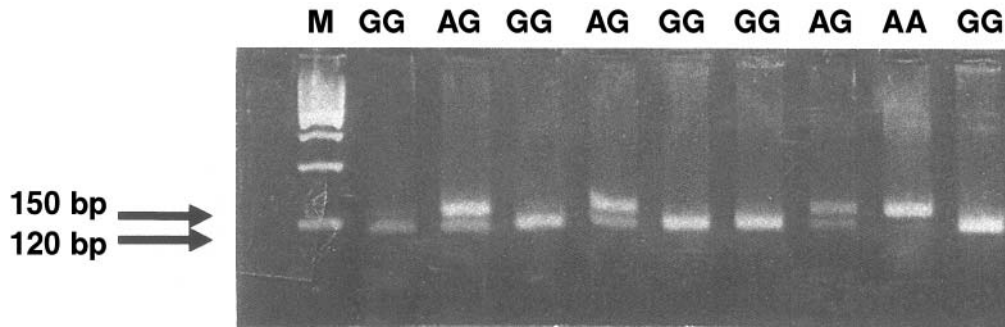
Figure 1. *EcoRI restriction pattern of the MMP-7 -181 A>G polymorphism, observed in nine patients. GG: high expression homozygotes; AG: heterozygotes; AA: low expression homozygote.*

carcinoma, and epithelial ovarian carcinoma (30-34). Furthermore, the G allele was associated with a smaller coronary artery diameter and increased risk of coronary disease in hypercholesterolemic patients (29).

In light of the above, we studied the *MMP-7* -181A/G polymorphism in patients with OSCC and healthy controls representing the general population in order to examine the possible association of this genetic variant with an increased risk and poor prognosis for this type of cancer.

Materials and Methods

The individuals under study were 279 unrelated Greeks (N=95) and Germans (N=184), consisting of 159 patients with OSCC and 120 healthy blood donors (controls) of similar gender, ethnicity and age. The patients were mostly men (N=120) and their age ranged between 40-84 years (mean 58.8±9.9 years). The gender ratio (N=71 men) and the age of controls (range 31-92 years; mean 58±17.8 years) were comparable to those of the patients.

The patients had developed oral cancer and had been operated on recently or up to a decade ago. In addition to clinical presentation and a family history concerning any type of cancer or thrombophilia, a biopsy with pathological diagnosis of tumor stages I-IV was available for each patient. Fifty-four patients (34%) had one or two first-degree relatives with any type of cancer, but their age range (mean=59.8 years) did not differ significantly from that of patients overall. Furthermore, thirty patients (18.9%) had one or two first-degree relatives with idiopathic thrombosis and were of an earlier age range (mean=58.5 years), but again with no statistical difference compared to the whole group. Fourteen patients (8.8%) had a positive family history for both cancer and thrombophilia (mean age=57.2 years).

Nearly all patients (93.6%) were smokers and about a third of them were alcohol abusers (32.05%). Two thirds of the controls (94%) reported abuse of tobacco and about one third, abuse of alcohol (47%). The two groups had similar dietary habits and almost all worked in a low-risk environment (with the exception of one patient and three controls who worked in chemical factories).

Blood samples were collected from patients and controls under study after informed consent. DNA was isolated with the use of a NucleoSpin™ kit (Macherey-Nagel GmbH & Co, Dóren, Germany). Molecular detection of the -181A/G polymorphism in

the *MMP-7* gene was performed using restriction fragment length polymorphism typing and gel electrophoretic analysis, as described elsewhere (35). The PCR conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 50 sec, 65°C for 1 min and 72°C for 50 sec and with a final elongation step at 72°C for 5 min. The primers used were: 5'-TGGTACCATAATGTCCTGAATG-3' (forward) and: 5'-TCGTTATTGGCAGGAAGCACACAATGAATT-3' (reverse). The generated PCR product of 150 bp was cleaved by the restriction enzyme *EcoRI* into two fragments of 120 bp and 30 bp only if the G allele was present (Figure 1). Several samples were studied twice for verification of obtained results.

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 Fisher's exact test and age-adjusted odds ratios, while all genotype distributions were checked for compliance with the Hardy-Weinberg estimates. In all statistical analyses concerning the number of relatives with a family history of cancer or thrombosis, it was assumed that all controls have nil values for the above variables (*i.e.,* all controls have a negative family history of cancer and thrombosis). Thus, odds ratios are most likely expected to overestimate the true likelihood of *MMP-7* genotypes and these variables. The calculation of all odds ratios with a 95% confidence interval (CI) was performed using the Maentel-Haenzel method using as referrent the homozygous "normal" low expression A/A genotype. A p-value of less than 0.05 was considered statistically significant.

Results

The prevalence of *MMP-7* -181A/G genotypes of healthy controls and patients with OSCC are shown in Tables I-III. All genotype distributions were in Hardy Weinberg equilibrium in the control group, as well as in the whole group and subgroups of patients. There were no significant differences of genotype and allele frequencies among the Greek and German controls, so the data were analyzed together. In the control group, the high expression G allele frequency observed was 42.5% (similar to other European populations), while the carrier frequency was 61.7%.

Table I. *Prevalence of MMP-7 A/G polymorphism in healthy controls and patients with oral cancer (total group of patients and subgroups according to cancer stage).*

Genotype	Controls	Patients			Patients with cancer stages I/II			Patients with cancer stages III/IV		
	(%)	(%)	*P*	OR (CI)	(%)	*P*	OR (CI)	(%)	*P*	OR (CI)
G/G	28 (23.3%)	27 (17%)	N.S.	1.11 (0.53-2.33)	16 (19.5%)	0.0418	2.62 (0.95-7.26)	8 (12.5%)	N.S	0.54 (0.21-1.39)
A/A	46 (38.3%)	40 (25.2%)			10 (12.2%)			28 (43.8%)		
A/G	46 (38.3%)	92 (57.9%)	0.0034	2.07 (1.17-3.67)	56 (68.3%)	<0.0001	4.56 (2.00-10.42)	28 (43.8%)	N.S.	0.93 (0.45-1.95)
Total	120 (100%)	159 (100%)			82 (100%)			64 (100%)		
Prevalenceof G allele										
G allele frequency	42.5%	45.9%	N.S.	1.10 (0.77-1.56)	53.7%	0.033	1.49 (0.98-2.28)	34.4%	N.S.	0.73 (0.46-1.15)
Carrier frequency of G allele	61.7%	74.8%	0.0257	1.64 (0.97-2.79)	87.8%	<0.0001	3.50 (1.58-7.76)	56.3%	N.S.	0.76 (0.40-1.46)

Fischer's *p*-value corresponds to genotype comparisons and allele frequency comparisons; odds ratios (OR) are age-adjusted; N.S.: not significant; CI: 95% confidence interval.

Table II. *Prevalence of MMP-7 -181 A/G polymorphism in healthy controls and patients with oral cancer according to family history of either cancer or thrombophilia.*

Genotype	Controls (%)	Patients with family history of cancer			Patients without family history of cancer			Patients with family history of thrombophilia			Patients without family history of thrombophilia		
		(%)	*P*	OR (CI)	(%)	*P*	OR (CI)	(%)	*P*	OR (CI)	(%)	*P*	OR (CI)
G/G	28 (23.3%)	10 (18.5%)	N.S.	0.92 (0.37-2.32)	14 (15.2%)	N.S.	1.32 (0.52-3.35)	2 (6.7%)	N.S.	0.51 (0.11-2.41)	22 (19%)	N.S.	1.60 (0.69-3.73)
A/A	46 (38.3%)	20 (37%)			18 (19.6%)			10 (33.3%)			28 (24.1%)		
A/G	46 (38.3%)	24 (44.4%)	N.S.	1.38 (0.6-3.2)	60 (65.2%)	0.0004	3.03 (1.53-6.01)	18 (60%)	N.S.	1.79 (0.69-4.67)	66 (56.9%)	0.0068	2.02 (1.05-3.90)
Total	120 (100%)	54 (100%)			92 (100%)			30 (100%)			116 (100%)		
Prevalence of G allele													
G allele frequency	42.5%	40.7%	N.S.	0.94 (0.58-1.53)	47.8%	N.S	1.20 (0.80-1.80)	36.7%	N.S.	0.75 (0.40-1.40)	47.4%	N.S.	1.23 (0.83-1.81)
Carrier frequency of G allele	61.7%	63%	N.S.	1.04 (0.50-2.15)	80.4%	0.004	2.27 (1.18-4.37)	66.7%	N.S.	1.19 (0.49-2.90)	75.9%	0.0245	1.77 (0.95-3.28)

Fischer's *p*-value corresponds to genotype comparisons and allele frequency comparisons; odds ratios (OR) are age-adjusted; N.S.: not significant; CI: 95% confidence interval.

The G allele frequency in the patient group (45.9%) was not significantly different from that in the controls (Table I). The same non-significant pattern was observed in all subgroups of patients in regard to categorization of family history, cancer stage, smoking or drinking habits, gender, age, and age at onset of oral cancer, with one exception (Tables I-III): A significantly higher G allele frequency (53.7%) was detected in the subgroup of patients in early stages of cancer in comparison to controls (*p*=0.033, Table I).

In contrast, the detected carrier frequency of the G allele was significantly higher in patients in comparison to controls (74.8% *versus* 61.7%, respectively, *p*=0.0257, Table I). The

Table III. *Prevalence of MMP-7 -181 A/G polymorphism in healthy controls and patients with oral cancer according to either alcohol consumption or smoking habits.*

Genotype	Controls (%)	Patients with tobacco abuse			Patients without tobacco abuse			Patients with alcohol abuse			Patients without alcohol abuse		
		(%)	P	OR (CI)	(%)	P	OR (CI)	(%)	P	OR (CI)	(%)	P	OR (CI)
G/G	28 (23.3%)	20 (14.7%)	N.S.	0.97 (0.44-2.14)	4 (40%)	N.S.	3.47 (0.57-21.28)	12 (25%)	N.S.	3.08 (0.91-10.38)	12 (12.2%)	N.S.	0.74 (0.31-1.75)
A/A	46 (38.3%)	36 (26.5%)			2 (20%)			8 (16.7%)			30 (30.6%)		
A/G	46 (38.3%)	80 (58.8%)	0.0067	2.01 (1.11-3.66)	4 (40%)	N.S.	1.56 (0.23-10.34)	28 (58.3%)	0.0051	2.17 (0.78-6.03)	56 (57.1%)	0.0492	1.74 (0.91-3.31)
Total	120 (100%)	136 (100%)			10 (100%)			48 (100%)			98 (100%)		
Prevalence of G allele													
G allele frequency	42.5%	44.1%	N.S.	1.03 (0.71-1.48)	60%	N.S.	2.10 (0.80-5.54)	54.2%	N.S.	1.64 (0.99-2.74)	40.8%	N.S.	0.91 (0.61-1.36)
Carrier frequency of G allele	61.7%	73.5%	0.0453	1.55 (0.89-2.69)	80%	N.S.	2.15 (0.41-11.32)	83.3%	0.0063	2.16 (0.83-5.58)	69.4%	N.S.	1.28 (0.71-2.31)

Fischer's *p*-value corresponds to genotype comparisons and allele frequency comparisons; odds ratios (OR) are age-adjusted; N.S.: not significant; CI: 95% confidence interval.

same pattern of significance was observed in the subgroups of patients with: a) early (I, II) stages of cancer ($p<0.0001$, Table I), b) without positive family history of cancer ($p=0.004$, Table II), c) without family history of thrombophilia ($p=0.0245$, Table II), d) with tobacco abuse ($p=0.0453$, Table III) and with alcohol abuse ($p=0.0063$, Table III). The observed significant difference in the carrier frequency of the G allele was due to the corresponding significant increase of A/G heterozygotes in the above-mentioned subgroups and total group of patients. In addition, A/G heterozygotes have a two-fold relative risk (odds ratio, OR) of developing oral cancer than do low expression A/A homozygotes in the whole group of patients and in almost all the above-mentioned subgroups (Tables I-III). The exception again is the subgroup of patients with early stages of cancer, in which A/G heterozygotes have a quadruple relative risk of developing oral cancer than do A/A homozygotes (Table I).

Discussion

MMP-7 is a protease that degrades the extracellular matrix facilitating invasion of the surrounding connective tissue by neoplastic cells (11-13, 18, 36, 37). Increased expression of MMP-7 has been observed in oral, esophageal, gastric, endometrial, colorectal and pancreatic ductal carcinoma (13, 20-26). A single nucleotide polymorphism (-181A/G) in the promoter region of the *MMP-7* gene influences its transcription levels, with the G allele corresponding to a

high gene expression (29). The frequency of the high expression G allele is reportedly higher in patients with several types of cancer, including esophageal, lung, gastric, colorectal and ovarian carcinoma (30-34).

In light of the above, the purpose of this study was to investigate whether the *MMP-7* -181A/G polymorphism might be associated with an increased risk for oral squamous cell carcinoma. The genotypes of patients with oral cancer were compared to those of healthy controls with matched age, gender and ethnicity. Despite the modest number of studied participants (N=279), a significant increase of high expression G alleles was observed in the subgroup of patients in early cancer stages (I,II), in comparison to controls. Furthermore, the carrier frequency of the G allele was significantly increased in the whole group of patients and their sugroups with early stages of cancer, without positive family history of cancer or thrombophilia, and with tobacco or alcohol abuse. The observed significant difference in the carrier frequency of the G allele was due to a corresponding significant increase of A/G heterozygotes in the above-mentioned subgroups and the whole group of patients. These data suggest that the high expression G allele exerts a dominant effect increasing risk in certain individuals. These findings are in accordance with previous reports in esophageal, non-small cell lung, gastric, ovarian, and colorectal cancer (30-32).

The observation that the percentage of G/G homozygotes was lower in the subgroups of patients might be due to both the relative increase of A/G heterozygous patients and the

fact that the studied sample was of advanced or middle age. It is known that individuals homozygous for the G allele that are hypercholesterolemic have a significantly increased risk for coronary disease and death due to thrombotic incidents (29), therefore such individuals might not have been included in the group of patients studied.

Based on the findings of this study, the high expression G allele of the *MMP-7* -181A/G polymorphism seems to be important only in early stages of oral cancer. Interestingly, similar results have been reported for esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma and non-small cell lung carcinoma (31). The G allele was associated with susceptibility for all three types of cancer, but not with lymph-node metastasis (31). These observations may be explained by the inhibiting contribution of MMP-7 to angiogenesis. MMP-7 has the ability to generate three major inhibitors of angiogenesis: angiostatin from plasminogen, as well as endostatin and neostatin-7 from collagen XVIII (17, 28). This notion is also in accordance with the reported high levels of MMP-7 only in early stages of intestinal carcinogenesis, implicating pathways activated very early in the tumor development sequence (27).

In the microenviroment of oral squamous carcinoma cells, secretion of MMP-7 is reportedly induced by cytokines, such as IL-8, TNF-α and IL-1β (4). In turn, MMP-7 may activate several MMPs, including proMMP-9, as observed in other carcinoma cells (38). By performing genetic association studies like the one presented here, we previously detected strong association of oral cancer with alleles resulting in high gene expression of IL-8 and TNF-α, regardless of their stage of tumor (10, 39), as well as high gene expression of MMP-9 only in early tumor stages (40). It seems that IL-8 and TNF-α are major contributing factors in increasing risk for OSCC, while high levels of MMP-7 and MMP-9 are essential only in the initial stages of oral tumorigenesis, due to their antiangiogenic roles (17, 28, 40).

As previously mentioned, the frequency of high expression G allele was found significantly higher in patients with esophageal cancer also, especially in smokers (31). However, this does not indicate that all mechanisms involved in the development of oral and esophageal cancer are identical. The latter conclusion is reinforced by the findings of two Chinese studies, in which a *MMP-1* gene polymorphism was found to be associated with oral cancer but not with esophageal cancer (41, 42).

In conclusion, the *MMP-7* polymorphism studied here was strongly associated with increased risk for oral cancer in certain individuals. As a consequence, it is of great importance to perform further genetic association studies regarding the contribution of factors related to angiogenesis, inflammation and thrombosis to predisposition to oncogenesis in the oral region. Any positive findings could ultimately result in the undertaking of preventive measures safeguarding the health status and lives of certain at risk individuals in the general population.

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