Strong association of the tissue inhibitor of metalloproteinase-2 polymorphism with an increased risk of oral squamous cell carcinoma in Europeans

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Abstract. The present study was performed in order to investigate the possible association of the -418 G/C polymorphism in the tissue inhibitor of metalloproteinase-2 (TIMP-2) gene, which affects its expression, with the risk of developing oral cancer. PCR-based restriction analysis was performed in DNA samples from 158 patients with oral squamous cell carcinoma (OSCC) and 168 healthy controls of equivalent sex, age and ethnicity (Greeks and Germans). Statistical analyses were performed with Fisher's exact test and the calculation of odds ratios with a 95% confidence interval (CI). The frequency of the low C allele expression was ten times greater in the patients than the controls (31% vs 2.7%, respectively; P<0.001). The C/C and G/C genotypes were associated with an increased risk of developing OSCC (P<0.001, OR=40.88, 95% CI=2.24-744.40, and P<0.001, OR=21.31, 95%=9.82-46.21, respectively). The same pattern of significant differences with the controls was also observed in the subgroups of patients in regard to the initial or advanced stages of oral cancer, family history of any type of cancer or thrombosis, and smoking habits or alcohol abuse. These findings are consistent with the reduced levels of TIMP-2 in the presence of the low expression C allele, which are insufficient to inhibit the matrix metalloproteinase-driven degradation of the extracellular matrix (leading to cancer invasion) and mitogen-driven neoangiogenesis (leading to tumor growth and metastasis). In conclusion, the studied TIMP-2 polymorphism is strongly associated with an increased risk of OSCC in Europeans carrying the low C allele expression. These results indicate that this polymorphism could serve as a genetic marker for the susceptibility of cancer in the oral cavity.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy and a major cause of cancer morbidity and mortality worldwide, with more than 300,000 new cases being diagnosed annually all over the world (1,2). This malignancy arises as a result of a multistep process in which several factors, such as environmental agents (smoking, alcohol, dietary habits and certain viruses) and genetic events (alterations in oncogenes and tumor suppressor genes) play an accumulating role (3). Recently, common inherited polymorphisms that are related to inflammation, angiogenesis and thrombosis have also been correlated with an increased risk of malignant tumors developing and progressing in the oral cavity (4-9). Such a factor, which is known to play an important role in angiogenesis and in the progression of OSCC, is the tissue inhibitor of metalloproteinase 2 (TIMP-2) (10-12).

TIMP-2 is a natural inhibitor of matrix metalloproteinase (MMP)-2, a member of a family of proteases involved in the degradation of the extracellular matrix (10). Moreover, TIMP-2 has a unique role in its ability to directly suppress the proliferation of endothelial cells *in vitro* and angiogenesis *in vivo* independently of the MMP-2 inhibition (10,11). In

addition, TIMP-2 is implicated in cell growth and apoptosis (13,14). Taking all these characteristics into account, several recent studies have reported a relationship between the increased TIMP-2 expression and the progression of certain malignancies including OSCC, serous ovarian carcinomas and urothelial bladder carcinomas (12,14-16).

A single nucleotide G/C polymorphism has been identified at position -418 in the promoter region of the TIMP-2 gene (17,18). The presence of the G allele has been shown to be associated with increased gene expression, possibly because it favours the binding of the Sp1 transcription factor on a consensus sequence in the promoter region of the TIMP-2 gene (19,20). The frequency of the C allele ranges between 10-20% in East Asians while in Caucasians it is considered to be much rarer. (18,20-24).

This polymorphism has been studied in Thai patients with head and neck squamous cell carcinoma. However, the results were inconclusive (20). Therefore, we investigated the possible association of the TIMP-2 -418 G/C gene polymorphism with an increased risk of oral cancer in European patients in comparison to healthy controls.

Materials and methods

The individuals under study were 326 Greeks and Germans, who participated after informed consent. The Departments of Oral and Maxillofacial Surgery of the Universities of Athens and Erlangen considered all the ethical aspects of this study and approved the protocol used. The studied individuals included 158 patients with oral squamous cell carcinoma and 168 healthy blood donors of equivalent ethnicity, age and gender. The age of the patients ranged between 40-80 years (mean age 58.6±10.2 years) and the age of the controls varied between 38-83 years (mean age 54.7±11.9 years). The studied individuals were mostly men: 79.7% of the patients (N=126) and 75% of the controls (N=126).

The patients included in this study had already developed oral cancer and had received surgical treatment within the last decade. In addition to a clinical presentation, a biopsy with a pathological diagnosis of tumor stages I-IV and a family history regarding any type of cancer or thrombosis, were available for each patient. Fifty eight patients (36.7%) had one or two first-degree relatives with cancer and their age range of 41-83 years (58.6±10.4 years) did not differ significantly from the whole group of patients. Furthermore, thirty two patients (20.3%) had one or two first-degree relatives with idiopathic thrombosis and an age range of 44-75 years (58±9.9), again with no statistical difference when compared to the whole group. Sixteen patients (10.1%) had a positive family history for both cancer and thrombosis with a mean age of 56.3±8 years. Nearly all the patients (93.7%) were smokers and about a third of them were alcohol abusers (32.9%). Most of the studied individuals 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 all the individuals under study and DNA was isolated with the use of the Nucleon™ kit (Amersham). Molecular detection of the -418 G/C polymorphism in the TIMP-2 gene was performed by restriction fragment length polymorphism typing. This involved a combination of PCR amplification and digestion with restriction endonuclease *Hga1* followed by gel electrophoretic analysis. The PCR conditions consisted of an initial denaturation step at 95˚C for 5 min, followed by 35 cycles of 94˚C for 55 sec, 55˚C for 1 min, and 72˚C for 55 sec, as well as a final elongation step at 72˚C for 7 min. The primers used were sense: 5'- GGATCCTGTCAGTTTCTCAA-3' and antisense: 5'- TTTCCCCTTCAGCTCGACTCT-3'. The generated PCR product of 176 bp was cleaved by the restriction enzyme, *Hga1,* into two fragments of 109 and 67 bp when the C allele was present, and while the G allele was not digested. For the verification of the molecular typing results, some of the samples were tested twice and their genotype was also confirmed by DNA sequencing.

The statistical analyses were performed using SAS® software (version 9.0; SAS Institute Inc.). The genotype distribution and allelic frequencies were analyzed with Fisher's exact test using the exact table P-values. The genotype frequencies of the whole group or subgroups of patients were compared to the respective genotypes of the control group. In all the statistical analyses, it was assumed that there were no patients in the control group with a family history of cancer, or thrombosis, nicotine or alcohol abuse. Thus, odds ratios are most likely expected to overestimate the true likelihood of the TIMP-2 genotypes and these variables. The age criterion for the adjustment of odds ratios has been set at 60 years. Similar frequency distributions regarding age are found in the respective genotypes between the 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 <0.05 was considered statistically significant. All the observed genotype and allele frequencies were analyzed according to Hardy-Weinberg, in order to avoid the possibility of potential bias.

Results

The obtained data of the detected TIMP-2 genotypes in the healthy controls and patients with OSCC are shown in Tables I-IV. The two studied European populations (Greeks and Germans) displayed no significant differences in the genotype and allele frequencies of the -418 G/C polymorphism, either among the controls or the patients (Table I). Therefore, the data for the two populations under study were analyzed together (Tables II-IV). All the observed genotype and allele frequencies did not decline from the Hardy-Weinberg equilibrium.

A highly significant difference in the G/C heterozygotes was observed between the oral cancer patients and the controls (54.4% vs 5.4%, respectively, OR=21.31, 95% CI=9.82-46.21, P<0.001). In comparison to the controls, who had a very low C allele frequency (2.7%) in accordance to previously studied Caucasian populations, the patients had a C allele frequency which was about ten times higher (31%, P<0.001).

This pattern of highly significant differences in the C allele and carrier frequencies in comparison to the controls was observed in all the subgroups of patients in regard to i) early or advanced cancer stages, ii) with or without positive family history of cancer, iii) with or without positive family

Table I. Prevalence of the TIMP-2 (-418G/C) polymorphism in the patients and healthy controls of Greek and German origin.

	Controls			Patients		
	Total	Greeks	Germans	Total	Greeks	Germans
Genotypes						
C/C	0 (0%)	0 (0%)	0 (0%)	6 (3.8%)	5 (4.8%)	1 (1.9%)
G/G	159 (94.6%)	107 (95.5%)	52 (92.9%)	66 (41.8%)	40 (38.5%)	26 (48.1%)
G/C	9 (5.4%)	5 (4.5%)	4 (7.1%)	86 (54.4%)	59 (56.7%)	27 (50%)
Total	168	112	56	158	104	54
Prevalence of T allele						
C allele freqency	9/336 (2.7%)	5/224 (2.2%)	4/112 (3.6%)	98/316 (31%)	69/208 (33.2%)	29/108 (26.9%)
Carrier frequency of C allele	9/168 (5.4%)	5/112 (4.5%)	4/56 (7.1%)	92/158 (58.2%)	64/104 (61.5%)	28/54 (51.9%)

The frequencies of the genotypes and C alleles are not significantly different among the two studied populations, either among the controls or the patients. TIMP-2, tissue inhibitor of metalloproteinase-2.

Table II. Prevalence of the TIMP-2 (-418G/C) polymorphism in the healthy controls and the total group of patients and their subgroups, in regard to the oral cancer stage.

Genotypes	Controls	Patients	Fisher's P-value	OR (CI)	Patients with cancer stages I & II	Fisher's P-value	OR (CI)	Patients with cancer stages III & IV	Fisher's P-value	OR (CI)
C/C	0 (0%)	6 (3.8%)	<0.001	40.88 (2.24-744.40)	4 (4.5%)	0.0014	78.12 (3.97-1537.73)	2 (2.9%)	0.0273	24.11 (1.12-519.46)
G/G	159 (94.6%)	66 (41.8%)		1 (referent)	36 (40.9%)		1 (referent)	30 (42.9%)		1 (referent)
G/C	9 (5.4%)	86 (54.4%)	<0.001	21.31 (9.82-46.21)	48 (54.5%)	<0.001	33.81 (13.70-83.41)	38 (54.3%)	<0.001	15.36 (6.50-36.31)
Total	168 (100%)	158 (100%)			88 (100%)			70 (100%)		
Prevalence of C allele										
C allele frequency	2.7%	31%	<0.001		31.8%	<0.001		30%	<0.001	
Carrier frequency of C allele	5.4%	58.2%	<0.001		59.1%	<0.001		57.1%	<0.001	

All odds ratios are age-adjusted. TIMP-2, tissue inhibitor of metalloproteinase-2; CI, confidence interval.

history of thrombosis, iv) smoking habits and v) alcohol abuse (Tables II-IV). It should be mentioned though, that the very low number of non-smoking patients (N=10) does not allow safe conclusions to be drawn in this subgroup. On the contrary, the fact that the C/C and G/C genotypes were found to be significantly increased among the individuals with and without alcohol abuse implies that the effect of TIMP-2 is not influenced by alcohol during oral oncogenesis.

Discussion

Higher levels of activated MMPs have been implicated in tumor development and metastasis (25). An important mechanism for the down-regulation of MMPs is via binding to the tissue inhibitors of metalloproteinases (TIMP-1 through TIMP-4), which belong to a family of homologous proteins (26). TIMP-2 is not only a natural inhibitor of

Table III. Prevalence of the TIMP-2 (-418G/C) polymorphism in the healthy controls and patients with oral cancer in regard to family history of either cancer or thrombosis.

Genotypes	Controls	Patients with family history of cancer	Fisher's P-value	OR (CI)	Patients without family history of cancer	Fisher's P-value	OR (CI)	Patients with family history of thrombosis	Fisher's P-value	OR (CI)	Patients without family history of thrombosis	Fisher's P-value	OR (CI)
C/C	0 (0%)	4 (6.9%)	<0.001	78.12 (3.97-1537.73)	2 (2%)	0.0495	24.11 (1.12-519.46)	0 (0%)	NC	NC	6 (4.8%)	<0.001	49.49 (2.71-904.76)
G/G	159 (94.6%)	22 (37.9%)		1 (referent)	44 (44%)		1 (referent)	16 (50%)		1 (referent)	50 (39.7%)		1 (referent)
G/C	9 (5.4%)	32 (55.2%)	<0.001	26.46 (10.50-66.69)	54 (54%)	<0.001	19.33 (8.38-44.59)	16 (50%)	<0.001	27.54 (9.19-82.56)	70 (55.6%)	<0.001	19.85 (8.91-44.24)
Total	168 (100%)	58 (100%)			100 (100%)			32 (100%)			126 (100%)		
Prevalence of C allele													
C allele frequency	2.7%	34.5%	<0.001		29%	<0.001		25%	<0.001		32.5%	<0.001	
Carrier frequency of C allele	5.4%	62.1%	<0.001		56%	<0.001		50%	<0.001		60.3%	<0.001	

All odds ratios are age-adjusted. TIMP-2, tissue inhibitor of metalloproteinase-2; CI, confidence interval; NC, non calculable P-value.

Table IV. Prevalence of the TIMP-2 (-418G/C) polymorphism in the healthy controls and patients with oral cancer in regard to either alcohol consumption or smoking habits.

Genotypes	Controls	Patients with tobacco abuse	Fisher's P-value	OR (CI)	Patients without tobacco abuse	Fisher's P-value	OR (CI)	Patients with alcohol abuse	Fisher's P-value	OR (CI)	Patients without alcohol abuse	Fisher's P-value	OR (CI)
C/C	0 (0%)	4 (2.7%)	0.0069	32.02 (1.68-611.23)	2 (20%)	0.0011	120.56 (5.02-2897.22)	2 (3.8%)	0.0119	63.82 (2.83-1438.99)	4 (3.8%)	0.0034	36.85 (1.92-705.76)
G/G	159 (94.6%)	62 (41.9%)		1 (referent)	4 (40%)		1 (referent)	18 (34.6%)		1 (referent)	48 (45.3%)		1 (referent)
G/C	9 (5.4%)	82 (55.4%)	<0.001	23.40 (10.69-51.21)	4 (40%)	0.0011	11.27 (1.53-83.14)	32 (61.5%)	<0.001	36.59 (13.51-99.13)	54 (50.9%)	<0.001	17.34 (7.65-39.31)
Total	168 (100%)	148 (100%)			10 (100%)			52 (100%)			106 (100%)		
Prevalence of C allele													
C allele frequency	2.7%	30.4%	<0.001		40%	<0.001		34.6%	<0.001		29.2%	<0.001	
Carrier frequency of C allele	5.4%	58.1%	<0.001		60%	<0.001		65.4%	<0.001		54.7%	<0.001	

All odds ratios are age-adjusted. TIMP-2, tissue inhibitor of metalloproteinase-2; CI, confidence interval.

MMP-2 but a suppressor of endothelial cell proliferation and angiogenesis as well (10,11). The complexity of the TIMP-2 functions indicates a possibly multiple role in cancer progression and metastasis (13,14,27). The levels of TIMP-2 have been correlated with the progression of OSCC, in addition to other carcinomas (12,14-16,27).

A single nucleotide polymorphism (-418G/C) in the promoter region of the TIMP-2 gene affects its transcription (20). Gene expression is lower when the less common C allele is present (18,20). In this light, the purpose of this study was to investigate the possible role of the -418G/C polymorphism in the risk of developing oral oncogenesis by comparing the TIMP-2 genotypes of patients with oral cancer and healthy controls of equivalent age, sex and ethnicity.

Despite the relatively small number of studied individuals, the overall obtained data revealed a strong association of the low expression C allele with an increased risk of developing oral cancer ($P<0.001$). Both the homozygous state C/C as well as the heterozygous G/C genotype were strongly associated with an increased risk of developing oral oncogenesis ($P<0.001$), indicating that the low expression C allele acts as a dominant genetic character. The association of the TIMP-2 polymorphism with oral cancer was so strong that no additive effect of the environmental factors that are known to be tumorigenic in the oral cavity (such as tobacco and alcohol) was observed.

These findings could be explained by the reduced amount of TIMP-2 in the presence of the C allele, which is insufficient to inhibit MMP-2 and mitogen-driven angiogenesis (10,11,20). MMP-2 is a protease involved in the degradation of the extracellular matrix and thus contributes to cancer invasion and metastasis, while neoangiogenesis is important for the viability, growth and metastastic potential of tumors (10,11,25).

The present study is not in accordance with another study conducted in a Thai population, concerning head and neck cancer (20). The distribution of the genotypes concerning the -418 C/G polymorphism in Thais was only borderline different ($P=0.059$) between the patients and the controls, while no significant difference was found among the C allele frequencies between the patients and the controls ($P=0.320$) (20). Nevertheless, only 46.4% of the Thai patients had OSCC (20). Therefore these results are not fully comparable with the findings of the present study. Diverse tumorigenic mechanisms possibly exist among tumors of the oral cavity and neck. This notion is reinforced by a number of studies, in which the polymorphisms in the MMP-1 or IL-8 genes have been associated with oral cancer but not esophageal cancer (9,28-30). Moreover, this discrepancy could be due to the fact that the frequency of the C allele is more common in the Thai controls (17.2%) and rare in Europeans (only 2.7%), indicating that there are differences in the distribution of genotypes among these ethnicities.

In conclusion, the studied TIMP-2 polymorphism is strongly associated with an increased risk of OSCC in Europeans carrying the low expression C allele. These results indicate that this polymorphism could serve as a genetic marker for the susceptibility to cancer in the oral cavity. Since other factors related to angiogenesis, inflammation and thrombosis have been also associated with this malignancy (7-9), further studies are necessary in order to clarify the role of all the potential genetic and environmental factors in the formation of OSCC, in an effort to safeguard the health status and lives of certain individuals who are at risk in the general population.

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