Angiotensinogen Polymorphism is Associated with Risk for Malignancy but not for Oral Cancer

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Abstract. *Background: In light of the recently found contribution of angiogenic and inflammation-related factors to malignancies, this study investigated the possible association of the angiotensinogen gene (AGT) with increased risk of oral cancer. Materials and Methods: The M235T polymorphism, which influences AGT gene expression, was evaluated by restriction fragment length polymorphism analysis in the DNA samples of 163 German and Greek patients with oral squamous cell carcinoma (OSCC) and 124 healthy controls of equivalent gender, ethnicity and age. Results: No significant difference of the mutant (235T) allele, which results in higher AGT gene expression, was observed in the whole patient group in comparison with the normal controls. Similarly, compared to the controls no significant difference of either allele or carrier frequency was detected in almost every subgroup of patients. Only in the subgroup of patients with a positive family history of cancer was a significant increase of mutant T allele and carrier frequencies observed, compared to the controls (50% vs. 36.7% and 79.3% vs. 61.3%, respectively, $p<0.05$ in both cases). In this particular subgroup of patients the odds ratio for OSCC of TT homozygotes was 3.57 (CI 95% 1.2-10.62), while for the MT heterozygotes it was 2.41 (CI 95% 1.06-5.49). Conclusion: This study did not reveal an association of the AGT M235T polymorphism with oral oncogenesis, but certainly suggested a possible association of this specific polymorphism with other types of cancer. The present findings support a previous suggestion that the pathway of oral oncogenesis is probably based on angiotensin-converting enzyme and bradykinin interaction and not on AGT and angiotensin peptides.*

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Oral squamous cell carcinoma (OSCC) is a common cancer, characterised by low survival rates and poor prognosis (1, 2). The multistep process of oral carcinogenesis is affected by various factors, such as tobacco and alcohol abuse, and by multiple genetic events such as mutations in oncogenes and tumor suppressor genes (2, 3). Recently, common polymorphisms in genes related to thrombosis, inflammation and angiogenesis have also been implicated in the predisposition for the development and advancement of oral cancer (4-11). This has encouraged further research on such factors with the hope that it might lead to better prevention and modification of the present low survival rate.

One such factor is angiotensinogen (AGT), a precursor of the angiotensin peptides and the only known naturally occurring rennin substrate (12, 13). The hydrolysis of AGT into angiotensin I, by rennin, is rate-limiting for the whole rennin-angiotensin system (RAS), which results in the production of the vasoactive peptides angiotensin II and III by angiotensin-converting enzyme (ACE) (12, 13). AGT is synthesized and secreted extracellularly by a variety of cells, most prominently hepatocytes, adipocytes and astrocytes (14). Even though, for many years AGT was thought to be involved in blood pressure control by simply being the unique substrate of rennin, recent studies have implicated AGT with inflammation as well as *in vitro* inhibition of human endothelial cell proliferation, cell migration and angiogenesis (15, 16). Based on the above mentioned AGT properties, the *in vivo* antitumoral effect of AGT has been investigated in recent studies, and it was found that AGT delivery by gene transfer could serve as a promising new strategy to block primary tumor growth and prevent metastasis (17).

The *AGT* gene is localized on chromosome 1q42-43, contains five exons and encodes a 61 kDa protein (12, 13). At least 15 molecular variants of AGT have been identified, but most studies have focused on a single amino acid substitution at position 235 because it is the only one known to influence its expression (18). A single base substitution in the 235 codon, results in threonine instead of methionine (M235T) (19). Studies of promoter function have revealed that this single base substitution influences the basal rate of gene transcription, providing a molecular mechanism for the association of the high expression T allele with increased hypertension and risk for thrombosis (19-21). Interestingly, some studies have reported that there are associations between the M235T polymorphism and sex-specific cardiovascular responses to exercise (22, 23). In addition, the blood pressure response is sensitive to both the genotype and the menopausal status in women (24). The prevalence of the high expression T allele is dependent on race and reaches 77.0% in blacks, 78.0% in Asians, but in whites ranges only between 34%-55% (25).

Despite the fact that the association of the M235T with hypertension and thrombosis has been well investigated, to our knowledge, only two studies have investigated this polymorphism in human neoplasias (26, 27). Based on the above, the possible correlation of the AGT M235T polymorphism with risk for oral cancer was investigated in a cohort of patients with oral cancer and healthy controls representing the general population.

Materials and Methods

The individuals under study were 287 Greeks and Germans, recruited after informed consent and approval of the ethical committees of the participating departments. They included 163 patients with oral squamous cell carcinoma and 124 healthy blood donors of similar age, ethnicity and gender.

The patients had developed oral cancer and had undergone surgery 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 in all cases. Fifty-eight patients (35.6%) had one or two first degree relatives with any type of cancer and their age range (41-83 years; 59±/10.2 years) did not differ significantly from the whole group of patients. Furthermore, thirty patients (18.4%) had one or two first-degree relatives with idiopathic thrombosis and their age range (44-75 years; 58.5±/10 years) was also not statistically different from the whole group. Fourteen patients (8.6%) had a positive family history for both cancer and thrombophilia (48-74 years; 57.2±/8.2 years).

DNA was isolated from blood with the use of a Nucleon™ kit (Amersham, Buckinghamshire, UK). The molecular detection of the M235T polymorphisms in the *AGT* gene was performed by restriction fragment length polymorphism typing of PCR products. The primers used were: Forward: 5'-CAGGGTGCTGTCC ACACTGGACCCC-3' and Reverse: 5'-CCGTTTGTGCAGGGCCT GGCTCTCT-3'. The PCR conditions consisted of an initial denaturation step at 94˚C, followed by 34 cycles of 94˚C for 50 sec, 59˚C for 60 sec, and 72˚C for 50 sec, as well as a final elongation step at 72˚C for 5 min. After treatment with restriction enzyme *Tth111* the PCR product of 165 bp remains intact in the presence of the M allele, while it is cleaved into two fragments of 141 bp and 24 bp in the presence of the T allele.

All the statistical analyses were performed using SAS® software (version 9.0; SAS Institute Inc, Cary, NC, USA). The frequencies of the 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 and age-adjusted odds ratios. All the observed genotype and allele frequencies were tested for compliance with Hardy-Weinberg equilibrium. The statistical analyses concerning the number of relatives with a positive family history of cancer or thrombosis, as well as nicotine and alcohol abuse assumed that all the controls had nil values for the above variables. Thus, the odds ratios are most likely expected to overestimate the true likelihood of AGT genotypes and these variables. 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

The prevalence of the detected *AGT* genotypes in healthy controls, the total group of patients and their subgroups in regard to family history of cancer are shown in Table I. All the M235T 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 two tested populations (Greek and German healthy controls) were analyzed together, since there were no significant differences of allele frequencies of the M235T polymorphism between the two populations. The observed genotypes in the control group were TT=15, MM=48, MT=61, resulting in a "mutant" T allele frequency of 36.7% (similar to other European populations) and carrier frequency of 61.3% (Table I).

The observed genotypes in the patient group (TT=23, MM=53, MT=87, Table I) and their mutant T allele and carrier frequencies (40.8% and 67.5%, respectively) were not significantly different compared to those in the control group. Similarly, in comparison to the controls no significant difference of either frequency was detected in the subgroups of patients: without a positive family history of cancer (TT=10, MM=36, MT=46, Table I); in early (I, II) stages of cancer (TT=14, MM=30, MT=38); in advanced (III,IV) stages of cancer (TT=8, MM=18, MT=42); with a positive family history of thrombophilia (TT=8, MM=10, MT=12); without a positive family history of thrombophilia (TT=14, MM=38, MT=68); with tobacco abuse (TT=20, MM=44, MT=76); without tobacco abuse (TT=2, MM=4, MT=4); with alcohol abuse (TT=6, MM=14, MT=30) or without alcohol abuse (TT=16, MM=34, MT=50). Interestingly, the only exception was the subgroup of patients with a positive family history of

Table I. *Prevalence of angiotensinogen M235T polymorphism in healthy controls and the total group of patients and their subgroups with regard to family history of cancer.*

Genotypes	Controls' No. (%)	Patients' No. (%)	Fisher's *p*-value	OR (CI)	Patients with family history of cancer (%)	Fisher's *p*-value	OR (CI)	Patients without family history of cancer (%)	Fisher's *p*-value	OR (CI)
Mutant homozygotes TT	15 (12.1%)	23 (14.1%)	N.S.	1.63 (0.73-3.66)	12 (20.7%)	0.036	3.57 (1.2-10.62)	10 (10.9%)	N.S.	1.21 (0.43-3.4)
Normal homozygotes MM	48 (38.7%)	53 (32.5%)		1 (referent)	12 (20.7%)		1 (referent)	36 (39.1%)		1 (referent)
Heterozygotes MT	61 (49.2%)	87 (53.4%)	N.S.	1.25 (0.73-2.13)	34 (58.6%)	0.047	2.41 (1.06-5.49)	46 (50%)	N.S.	0.95 (0.51-1.78)
Total	124 (100%)	163 (100%)			58 (100%)			92 (100%)		
Prevalence of mutant T allele										
T allele frequency	36.7%	40.8%	N.S.	1.25 (0.87-1.78)	50.0%	0.022	1.93 (1.2-3.12)	35.9%	N.S.	1.04 (0.68-1.58)
Carrier frequency of T allele	61.3%	67.5%	N.S.	1.31 (0.79-2.19)	79.3%	0.018	2.47 (1.16-5.29)	60.9%	N.S.	1.01 (0.56-1.83)

N.S. : not significant p-value.

cancer, in which a significant increase of the mutant T allele and carrier frequencies was observed, compared to controls (50% and 79.3% respectively, $p<0.05$ in both cases, Table I). In this particular subgroup of patients both TT homozygotes and MT heterozygotes were significantly increased compared to the controls (Table I). The relative risk (odds ratio) for OSCC of the TT homozygotes was 3.57 (CI 95% 1.2-10.62), while for MT heterozygotes it was 2.41 (CI 95% 1.06-5.49). Finally, there were no major differences due to gender, age, or age at onset of oral cancer.

Discussion

Despite the relatively small sample of studied individuals, the overall data of this study revealed no strong association of the M235T polymorphism with an increased risk for oral squamous cell carcinoma. Compared to the controls, the prevalence of the "mutant" high expression T allele was not significantly different in the total group of patients or in any of their subgroups, except one.

The fact that an increase in high expession T allele frequency was observed only in the subgroup of patients with a positive family history of cancer may indicate a correlation of the M235T polymorphism with other types of cancer, but only a subtler association specifically with oral cancer. Despite the recent reports attributing tumor-related properties to the M235T polymorphism, only two studies have investigated its association with human neoplasias both of which involved women and produced inexplicit findings (26, 27). Homozygocity for the low expression M allele was associated with an increased risk for breast cancer in postmenopausal women, while the presence of the high expression T allele was incriminated in the development of polycystic ovary syndrome (26, 27).

The findings of the present study, which suggested that oral oncogenesis is not associated with increased levels of AGT, are in accordance with our previous report that the low expression allele of the ACE I/D gene polymorphism was associated with risk for oral cancer (28). Low levels of ACE lead to minimal production of angiotensin peptides, therefore, it seems that oral carcinogenesis does not follow the classical RAS-driven oncogenic pathway, through the increased production of angiotensin peptides. Alternatively, a bradykinin-related pathway is proposed in the case of oral carcinogenessis. A well

established role of ACE is the inactivation of bradykinin, which is known to contribute to tumor formation through its ability to stimulate growth and increase vascular permeability (29). It follows that the low expression of ACE in oral cancer patients would correlate with reduced inactivation of bradykinin and, therefore, in this case oncogenesis could be driven through a pathway based on ACE-bradykinin interaction and not through AGT and angiotensin peptides. This notion of a more sensitive bradykinin-related reaction in the oral cavity, in contrast to other tissues, is also supported by the fact that patients treated with ACE inhibitors often present with angioedemas exclusively in the oral region, due to the increased local levels of bradykinin (30).

In conclusion, the findings of the present study indicate that the M235T polymorphism of the *AGT* gene is not important in oral oncogenesis, but might as well be associated with other types of cancer. Therefore, it would be interesting to perform genetic association studies in order to assess the contribution of this polymorphism in other malignancies.

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