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A common variant of the angiotensinogen gene and the risk of coronary artery disease in a German population

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The thymidine to cytosine transition at position 704 in exon 2 of the angiotensinogen gene leads to the amino acid substitution of threonine for methionine (T235 variant) and is responsible for elevated plasma levels of angiotensinogen. To examine the influence of T235 on the risk of coronary artery disease (CAD) we genotyped 184 CAD patients, 77 controls in whom CAD was excluded angiographically, and 155 healthy controls without signs of CAD by polymerase chain amplification and restriction enzyme digestion. Allele frequencies for A (wildtype) and a (mutant allele) in the total study population were 0.538 and 0.462, 0.536 and 0.464 in the healthy controls, and 0.481 and 0.519 in patients with excluded CAD, respectively. The allele frequencies and the genotype distribution in these groups did not show a significant difference. In conclusion, we did not observe an association between the T235 variant of the angiotensinogen gene and the risk of CAD.

1. Introduction

The systemic and local renin angiotensin (aldosterone) systems (RAAS) play an important role in the pathophysiology of several cardiovascular diseases [1, 2]. Activity of circulating effectors of the RAAS is under tight genetic control [3, 4]. There is great continuing interest in the link between genetic variants of components of the RAAS and cardiovascular diseases. A considerable amount of studies showed that the insertion/deletion (I/D) polymorphism of the angiotensin I converting enzyme (ACE) gene [5] is associated with an increased risk of cardiovascular diseases [3–9]. Gene variants of the angiotensin II receptor type 1 (AT_1)-receptor gene have been found to interact with the ACE genotype to increase the risk of myocardial infarction [9] and have been associated with hypertension [10]. The thymidine to cytosine transition at position 704 in exon 2 of the angiotensinogen (Ao) gene, which is responsible for the amino acid substitution of threonine for methionine (T235) at the protein level, is responsible for elevated plasma levels of angiotensinogen (Ao) [11]. Increased serum concentrations of ACE are associated with an increased risk of coronary artery disease (CAD) [12]. The plasma concentration of Ao influences the level of angiotensin II by beeing substrate for the protease renin. Cleavage of Ao by renin is known to be the rate limiting step in the RAAS [13]. The T235 variant of Ao has been shown to be associated with an increased risk of arterial hypertension in a French and a Japanese population study [11] as well as with an increased risk of coronary artery disease in a white population of New Zealand [14]. Badenhop et al. [15] found a significant association between this angiotensinogen polymorphism in children and grandparental CAD. Other studies failed to show any correlation between T235 and hypertension [16]. In addition, Hingorani [17] showed that T235 variant of Ao does not appear to be a marker for blood pressure variation in a group of 125 individuals with essential hypertension but found that T235 contributes to individual differences in the blood pressure response to ACE inhibition.

In the light of these contradicting reports, the aim of this study was to compare the T235 angiotensinogen genotype distribution in patients with angiographically proven CAD to that of a control group in order to assess the cardiac risk associated with this polymorphism.

2. Investigations and results

After PCR and restriction enzyme digestion agarose gel electrophoresis yielded at 186 bp product if the wildtype (A) and a 141 bp and a 45 bp product if the mutant (a) allele was present. Figure 1 shows a representative agarose gel in which PCR products amplified from human genomic DNA followed by restriction enzyme digestion have been resolved. The three possible genotypes were designated *AA*, *Aa* and *aa*. The *a* allele indicates the thymine-cytosine transition.

The corresponding genotype frequencies (see also Table 1) were 29.7% for *AA*, 47.7% for *Aa*, and 22.6% for *aa* in healthy control subjects (control group 1); 24.7% for *AA*, 58.4% for *Aa*, and 16.9% for *aa* control group 2 (patients, whom CAD was excluded angiographically), and 30.4% for *AA*, 54.9% for *Aa*, and 14.7% for *aa* in the coronary artery disease group. The corresponding genotype frequencies were 25.0% for *AA*, 57.5% for *Aa*, and 17.5% for *aa* in the entire study population. Allele frequencies for *A* (wildtype allele) and *a* allele (mutant allele) were 53.6% and 46.4% in the healthy controls, 48.1% and 51.9% in patients with angiographically excluded coronary artery disease, and 58.2% and 41.8% in coronary artery disease cases, respectively. The allele frequencies of the total study population were 53.8 and 46.2. Genotype frequencies did not differ significantly from the frequencies predicted based on the Hardy-Weinberg equilibrium in healthy controls ($\chi^2 = 0.2409$, $p = 0.8865$), control group 2 ($\chi^2 = 2.486$, $p = 0.2885$), cases ($\chi^2 = 3.1349$, $p = 0.2086$), and the entire study population ($\chi^2 = 0.8376$, $p = 0.6578$). This relative risk of coronary artery disease was not increased in individuals with the *aa* genotype (odds ratio 0.8, 95% CI 0.71 to 1.1, $p = 0.7051$).

3. Discussion

Individuals with *aa* genotype have been reported to have higher angiotensinogen blood concentrations [19]. Although angiotensinogen is mainly synthesized in the liver, many tissues are able to express angiotensinogen mRNA as well. Angiotensinogen as a substrate for renin is known to be an important link in the RAAS involved in the systemic control of fluid volume and blood pressure as well as to be an important regulating factor at the tissue

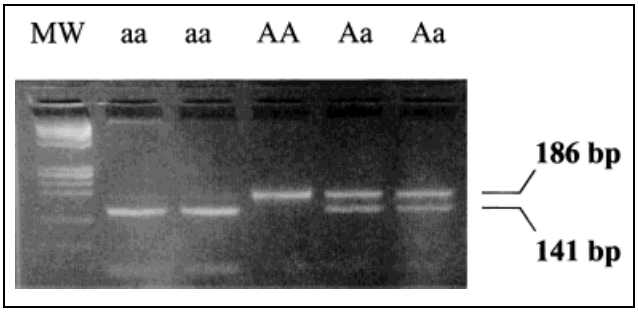

Fig. 1: The Figure shows a representative agarose gel in which PCR products amplified from human genomic DNA have been resolved. The homozygous methionine allele of the angiotensinogen gene M235 appears as a single 186 bp band and T235 as digested 141 band (the cleaved 45 bp run off the gel). The Ao gene molecular variants were designated *AA*, *Aa* and *aa*. The *a* allele indicated the thymine-cytosine transition

level [20, 21]. Angiotensin II production is sensitive to small changes in angiotensinogen concentrations in circulation [21]. The range of hemodynamic and local effects of an activated RAAS is compatible with the concept of an increased risk for the development of CAD conferred by the T235 variant of the Ao gene.

In this study however, we did not observe an association of the T235 variant of the Ao gene with an increased risk of CAD in our study population. How can we explain the discrepancy in results of the present study and earlier reports from Japan [11] and New Zealand [14]? The most likely reasons are differences in the criteria used to select the patients and controls and differences in the genetic background of the recruited individuals.

Investigations of the T235 variant of Ao gene in the Japanese population revealed a much higher amount of *aa* individuals varying between 49 and 79% [19, 22, 23]. Out of these three studies in Japanese populations, only Ishigami et al. [19] investigated the association of the *aa* genotype with CAD, reporting an excess of the homozygote T235 variant in the CAD group. Hata et al. [22] found a positive association of T235 variant with hypertension, and Nishiuma et al. [23] with carotid atheroma, respectively. Studies conducted in European and Northern American populations revealed an amount of homozygote mutants between 12 and 22%. Katsuya et al. [14] recruited in 1992 and 1993 patients discharged after treatment or evaluation for CAD between 1989 and 1993, thus, none of those who died in the period between evaluation and blood sample collection (a period that covers up to 4 years) are represented. In our study the recruitment of patients and blood sample collection in patients was done simultaneously with coronary angiography. However, it has to be considered that a selection bias possibly might have been introduced in our study. In addition, our study did not include people with fatal myocardial infarction (MI) and post MI patients not undergoing coronary angiography. Hence, because the genotype distribution in our study population did not deviate significantly from the Hardy-Weinberg equilibrium a selection bias is not very likely.

The most likely reason for the absence of an association between the T235 variant of Ao and the risk of CAD in our population is a different allelic distribution of this mutation in the German population. Although it is not impossible that the T235 variant of Ao itself is the pathophysiological relevant disease-causing alteration, it has to be considered that the transition in nucleotide 704 at exon 2 is in linkage disequilibrium with a mutation elsewhere on the Ao gene. In this case the T(704) to C transition could serve as a genetic marker. A different genetic background in this population may account for a decline in the degree of the linkage equilibrium between the marker and the putative disease mutation [24].

Also, it is important to recognize that studies of linkage disequilibrium are highly sensitive for the selection of an appropriate control sample [24]. In our study all patients and a part of our controls underwent coronary angiography. So a proper diagnosis could be established and CAD could be excluded in a remarkable part of the controls with high safety. In order to avoid a selection bias, because the individuals of the control group underwent corronary angiography for evaluation of angina like chest pain in association with standard risk factors, we genotyped a group of apparently healthy volunteers without clinical signs of CAD, additionally.

Furthermore, our study may be limited by sample size. Case and control group may have been too small to pick up significant differences. Even the small number of female patients among the case patients could be an important reason for underepresentation of the T235 variant. In

Table 1: Distribution of the *AA*, *Aa* and *aa* genotype

	Ao genotype, %			p	Odds ratio (95% CI)
	AA	*Aa*	*aa*		
Total population	25.0	57.7	17.5		
Predicted frequencies	28.7	49.7	21.5	0.6578	
CAD (group 1)	30.4	54.9	14.7	0.3928	0.8 (0.71 to 1.1)
Control group 2	29.7	47.7	22.6		
Control group 3	24.7	58.4	16.9		

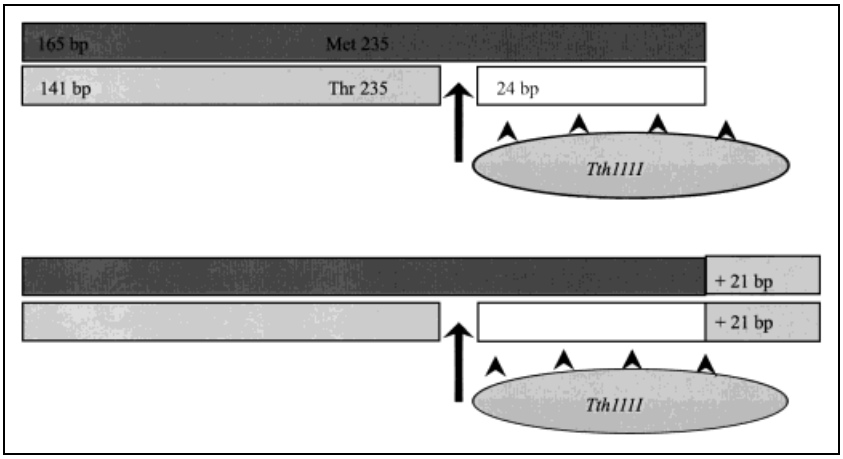

Fig. 2:
The PCR-product resulting from amplification with the extended primer offers better adhesion of the restriction enzyme *Thh1111* (arrowheads) and digestion (arrow) facilities

Table 2: Epidemiological and biochemical data (mean ± SD)

	CAD (group 1)	no CAD (group 2)
n	184	77
Sex (%male)	69.3	84.5
Age (years)	56.5 ± 10.6	59.9 ± 9.2
Body mass index (kg/m^2)	26.6 ± 3.5	26.9 ± 3.1
Hypertension (%)	37.0	49.3
Total Cholesterol (mmol/l)	6.2 ± 1.2	6.6 ± 1.5
LDL Cholesterol (mmol/l)	4.0 ± 1.1	4.4 ± 1.3

* = $p < 0.05$

American and French study populations T235 variant was more prevalent among female hypertensives than in male patients [11].

Methodical aspects also have to be considered to influence the accuracy of the findings in large-scale genotyping studies. We received proper bands and well reproducible results using the mismatch-primer PCR-amplification published by Russ et al. [18] only after introducing an extended downstream primer to facilitate the adhesion of the restriction enzyme in the amplified sequence. Therefore, was suggest the use of this altered assay in further studies investigating the T235 variant of Ao to get comparable and reproducible results.

The present data implicate that in a German population the T235 variant of Ao, which is known to be associated with elevated Ao levels does not serve as an useful indicator for an increased risk of CAD. So far there are conflicting results reported in the literature as discussed above. A large prospective study could help to clarify this issue.

Further data are needed to gain more information about the relations of genetic variants of the different parts of RAAS, their influence on the functional status of RAAS and their consequences including the risk of CAD.

4. Experimental

4.1. Patients and study design

Our study population consisted of 261 consecutive individuals (162 male and 99 female) who underwent coronary angiography at our institution either because of symptoms relating to coronary artery disease or for known coronary artery disease and 155 healthy controls. The protocol of this study has been approved by the Ethics Committee of the University of Jena. Blinded assessment of coronary angiograms of 261 patients was done by cardiologists not involved in this study. A coronary stenosis of ≥50% luminal diameter was considered to be a "significant lesion" and established the diagnosis of coronary artery disease. To qualify for inclusion, CAD patients (group 1) were required to have at least one obstruction of a major coronary artery ≥50% of luminal diameter with or without prior myocardial infarction. The second group (group 2) comprised 77 patients in whom coronary angiography revealed no coronary artery wall abnormalities. The control group (group 3) comprised 155 individuals (mean age 55.8 ± 13.9 years, 103 male and 52 female) who did not have a history of angina or myocardial infarction, who were selected randomly from the local registry office. Additional risk factor data were not available for these healthy controls. The patients demographic and common risk factor data are summarized in Table 2.

4.2. Angiotensinogen genotype determination

DNA was extracted from peripheral blood leukocytes using a commercially available extraction method (Immucor, Rödermark, Germany). Ao genotypes were identified by mismatch-primer PCR-amplification based on the method described by Russ et al. [18], with some modifications. To optimize the digestion by *Tth111*, we used the reported upstream primer 5′CCg TTT gTg Cag ggC Ctg gCT CTC T 3′, but lenghtened the downstream primer (additional sequence indicated by italic letters, restriction site underlined): 5′*CgC AAg TgA AAT CTC CTC CgT* CAg ggT gCT gTC CAC ACT ggA CCCCC 3′. Aim of the extension was to facilitate the adhesion of the restriction enzyme in the amplified sequence (Fig. 2).

PCR was performed over 40 cycles of 50 s denaturation at 93 °C, 1 min annealing at 62 °C, and 30 s extension at 72 °C. The PCR product of the mutant gene carries a half site for *Tth111 I* or its isochizomer *AspI*. PCR products were digested using *Tth111 I* at 37 °C for 12 h and separated by agarose gel (4.6%) electrophoresis and ethidium-bromide stained.

PCR results were scored blinded with regard to case-control status. Double determination of the genotype in randomly selected specimens was done to check the reproducibility of the results.

4.3. Statistical analysis

The allele and genotype frequencies among cases and control subjects were counted and compared by χ^2 test with Hardy-Weinberg predictions. Odds ratios with 95% confidence intervals and two-tailed p values were calculated as a measure of the association of the angiotensinogen genotype with the presence of coronary artery disease. The baseline values of the groups were compared by unpaired t-test. Statistical tests were performed using SPSS software package.

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