Gene polymorphisms of ACE and the angiotensin receptor AT2R1 influence serum ACE levels in sarcoidosis


1 Fraunhofer-Institute for Toxicology and Experimental Medicine, Clinical Airway Research, Hannover, Germany; 2 Medical Dept. I, Herford General Hospital, Herford, Germany; 3 Medical Hospital, Research Center Borstel, Borstel; 4 Department of Pneumology, University Medical Center, University of Freiburg

Abstract. Angiotensin converting enzyme (ACE) is thought to influence susceptibility, disease progression, and/or outcome of sarcoidosis by functional mutations/polymorphisms of the ACE gene, such as the ACE gene deletion/insertion (D/I) polymorphism or the angiotensin receptors like the angiotensin II receptor type 1 (AT2R1) A1166→C polymorphism. The aim of our study was to examine the distribution of the ACE D/I genotypes and the AT2R1 A1166→C genotypes in sarcoidosis and healthy controls, and to test their influence on disease progression. In this study, we assessed ACE and AT2R1 genotypes by PCR in 264 healthy Caucasians and 95 sarcoidosis patients. Serum ACE levels were determined using a kinetic test. Genotyping sarcoidosis patients for the AT2R1 A1166→C polymorphism revealed an increase in homozygous genotypes CC (sarcoidosis: 11.6%, controls: 9.2%) and AA (sarcoidosis: 61.1%, controls: 47.3%) but a lower frequency in heterozygous genotypes (sarcoidosis: 27.4%, controls: 43.5%; p=0.024) which was more pronounced in male patients. The co-incidence of DI and AC was less frequent in patients with sarcoidosis, suggesting protection by the combination of DI and AC. The AT2R1 A1166→C gene polymorphism modulated the effect of the ACE D/I polymorphism on serum ACE levels with the A allele promoting its influence and the C allele reducing it. We conclude that neither the ACE D/I nor the AT2R1 A1166→C polymorphism has a role in sarcoidosis disease progression. In males, the homozygous AT2R1 genotypes CC and AA possibly increase the risk for sarcoidosis. Co-incidence of the heterozygous genotypes DI and AC might be protective against sarcoidosis. (Sarcoidosis Vasc Diffuse Lung Dis 2009; 26: 139-146)

Key words: angiotensin-converting enzyme, Angiotensin II type 1 receptor, sarcoidosis, gene polymorphism

Introduction

Sarcoidosis is a chronic systemic granulomatous disease of unknown etiology characterized by accumulation of activated T-lymphocytes and mononuclear phagocytes forming non-caseating epitheloid granuloma in the affected organs.

High serum angiotensin converting enzyme (ACE) levels have been reported in patients with untreated active sarcoidosis (1), and spontaneous or corticosteroid-induced remission can be heralded by decreasing serum ACE values (2). Therefore, it is imaginable that the system of signal transduction initiated by the cleavage of angiotensin by ACE is involved in the pathogenesis of sarcoidosis, influencing susceptibility, disease progression, or disease outcome. Among the possible mechanisms are functional polymorphisms of either the ACE gene or its receptors.
The identification of a deletion/insertion (D/I) polymorphism in the ACE gene (chromosome 17) with serum ACE levels corresponding to ACE D/I genotypes in the order II<DI<DD (3) encouraged several studies investigating the influence of this variation on sarcoidosis susceptibility or outcome. So far, controversy persists with some studies not finding an association between ACE D/I polymorphism and incidence or severity of sarcoidosis (4–6). Others, however, identified the D allele as a possible risk factor for sarcoidosis in Japanese women (7) and in an African-American population (8). In a study in Germany, there was an over-representation of the ACE-genotype DD in patients with sarcoidosis and their families, suggesting that genotype DD might be a promoter to clinical manifestation of the disease (9). Disease progression correlated with the D allele in a Finish population (10), whereas in African-Americans, it seemed to be promoted by the I allele (8).

The detection of polymorphisms in the angiotensin II receptor type 1 gene (chromosome 3) (11) led to the assumption that there might also be a link between these receptor mutations, the serum ACE levels, and the course of sarcoidosis. The Angiotensin II Type 1 Receptor (AT2R1) A_{1166}→C gene polymorphism represents the transition from A_{1166} to C, which is in a non-translated region and may therefore be non-functional. However, Takemoto et al. found a higher serum ACE activity in sarcoidosis patients carrying the C allele of the AT2R1 gene (12). In the same study, no difference was found in the allele frequency of the AT2R1 A_{1166}→C gene polymorphism in patients with sarcoidosis and healthy controls (12). So far, the impact of the AT2R1 A_{1166}→C gene polymorphism on disease progression in sarcoidosis has never been investigated.

There are also studies focusing on possible interactions of the ACE gene polymorphism and the AT2R1 gene polymorphism in order to better understand the contribution of these polymorphisms in polygenetic conditions. In a large study in male Caucasians, no interaction between the AT2R1 A_{1166}→C gene polymorphism and the ACE D/I gene variation was found with regard to the risk of coronary artery disease or myocardial infarction (13). Others, however, found a significant synergism with an increased risk of myocardial infarction in patients carrying both the ACE DD genotype and the C allele of the AT2R1 gene (14–16). According to these data, the interaction between the genotypes of ACE and AT2R1 seems to modify the phenotype (disease process) in coronary atherosclerosis, contributing to inter-individual differences in the severity of the disease (16).

The aim of our study was to examine the distribution of the ACE D/I genotypes as well as the AT2R1 A_{1166}→C genotypes in a population in northern Germany both in sarcoidosis and healthy controls to identify a genetic risk factor for sarcoidosis. Second, the association of the ACE D/I gene polymorphism and the AT2R1 A_{1166}→C gene polymorphism with sarcoidosis disease progression was investigated. We were especially interested in possible interactions between the ACE and the AT2R1 genotypes affecting the sarcoid phenotype. Finally, the effect of the AT2R1 A_{1166}→C gene polymorphism on serum ACE levels was analyzed.

**Materials and Methods**

**Subjects**

264 healthy volunteers (90 male, 174 female; age 37 ± 12 years) and 95 patients with sarcoidosis participated in the study (54 male, 41 female; age 43 ± 13 years). All participants were Caucasians recruited in the region of Northern Germany. The radiological type 0 (normal chest X-ray) was present in 5.3% of the sarcoidosis patients, whereas 14.7% of patients were diagnosed with type I (bihilar adenopathy, normal lung parenchyma), 51.6% with type II (bihilar adenopathy, reticular-nodular pattern), 18.9% with type III (no adenopathy, reticular-nodular pattern), and 9.5% with type IV sarcoidosis (pulmonary fibrosis) (17).
the last 12 months, (ii) indication for therapy with other immunosuppressive drugs such as azathioprine or methotrexate within the last 6 months, or (iii) progressive loss of lung function with limitations of at least two of the following parameters: vital capacity (VC) < 70% of predicted, total lung capacity (TLC) < 70% of predicted, CO-transfer < 50% of predicted, and PaO₂ < 70 mmHg (n = 18, observation period 11 ± 5 months) (Table 1).

The healthy controls were employees who volunteered for donating blood after an uneventful examination by the occupational medical service of the Research Center Borstel.

None of the healthy controls or the sarcoidosis patients was on any medication containing ACE-inhibitors. Informed consent was obtained from all participants and the study was approved by the responsible Ethics Committee of the Medical University Lübeck.

**DNA extraction and genotyping**

DNA was extracted from anticoagulated venous blood samples by phenol precipitation followed by digestion with proteinase K using Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA) and used for the PCR reactions.

For the detection of the AT2R1 A1166→C polymorphism the first step was PCR in a 25 µl mixture consisting of 2.5 mM MgCl₂, 5 pmol of each primer (primer 1: 5' TGG GAC CAC AGC GCC CGC CAC TAC 3'; primer 2: 5' TCG CCA GCC CTC CCA TGC CCA TAA 3'), 0.2 mM of each deoxyribonucleoside triphosphates (dNTPs) (Invitrogen), 0.5 U of Taq polymerase (Invitrogen), 5% of dimethyl sulfoxide (DMSO, Sigma, Munich, Germany), and 2.5 ml 10xPCR-Buffer (Invitrogen). The PCR was run as follows: five minutes initial denaturation at 95°C, followed by 40 cycles of 30 seconds (s) at 94°C (denaturation), 45 s at 53°C (annealing), and 45 min at 72°C (extension), followed by final elongation step at 72°C for 5 min. After PCR the products were digested by DdeI (2U; Invitrogen) in the respective buffer at 37°C for 6 h or over night. In case of a C-allele the PCR product will be cut in 2 fragments of 211 and 139 bp whereas the A-allele is left untouched resulting in a single fragment of 350 bp. Heterozygotes are characterized by the presence of all three bands.

The respective fragment in intron 16 of the ACE gene was amplified according to the method of Rigat et al. (18) as previously described (19). Briefly, DNA was amplified in a 25 µl mixture consisting of 2.5 mM MgCl₂, 5 pmol of each primer (primer 1: CTG GAG ACC ACT CCC ATC CTT TCT, primer 2: GAT GTG GCC ATC ACA TTC GTC AGA, (MWG, Biotech AG, Ebersberg, FRG), 0.2 mM of each deoxyribonucleotide triphosphates (dNTPs) (Invitrogen, Carlsbad, USA), 0.5 U of Taq polymerase (Invitrogen), and 2.5 ml 10xPCR-Buffer (Invitrogen). PCR settings were the same as above with the exception of 53°C annealing temperature. The presence of D allele resulted a 192 bp amplicon, and the I allele could be detected by an 479 bp PCR product. Due to the preferential amplification of the D allele in heterozygous samples all samples found to have the DD genotype were included in a second insertion-specific PCR (ACE-INS-PCR; ACE-INS-F: TGG GAC CAC AGC GCC CGC CAC TAC; ACE-INS-R: TCG CCA GCC CTC CCA TGC CCA TAA). The reaction buffer contained 5% DMSO (SIGMA-ALDRICH, Munich, Germany) and 1.5mM MgCl₂. The PCR conditions were as follows: five minutes initial denaturation; 30 cycles:

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30s denaturation 94°C, 45s annealing 62°C, 40s extension 72°C, 5min. final elongation 72°C. The presence of an I allele resulted a 335 bp PCR product whereas for samples homozygous for DD no products could be detected (20).

**Serum ACE measurement**

Serum ACE was determined photometrically with a kinetic test (Bühlmann ACE Kinetic Kit, Bühlmann Laboratories, Allschwil, Switzerland). The tests were performed according to the manufacturer’s instructions.

**Statistical methods**

Statistical analysis was performed with the \( \chi^2 \)-Test or the Mann-Whitney-U-Test using StatView 5.0 software (SAS Institute, NC). Partly the data are presented as boxplots depicting the median, the 25th and 75th percentile, the 10th and 90th percentile as well as single values below the 10th and above the 90th percentile. Allele frequencies were calculated by the following formula: percentage = sum of alleles/(2x number of individuals) x 100. All comparisons performed in this study were single comparisons using Yates corrected chi-square test. To compare the degree of association between the given genotypes or alleles for the given groups odds ratios (OR) were calculated with confidence intervals (CI) of 95%. The correlation between different parameters was analyzed using Spearman’s rank correlation coefficient (rs). P-values of less than 0.05 were considered as significant. Linkage disequilibrium analysis was performed as previously described (21).

**Results**

**Distribution of ACE D/I genotypes**

The distribution of the D/I polymorphism in the ACE gene in our study population was determined by PCR (Figure 1). In the entire sarcoidosis cohort (n = 95), the homozygous genotype DD was found in 31.6% of the subjects (allele frequency: 20%

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**Fig. 1.** Distribution of ACE D/I genotypes and AT2R1 A1166→C genotypes in sarcoidosis and healthy controls. Percentages of ACE D/I and AT2R1 A1166→C genotypes are shown for the entire sarcoidosis cohort (n = 95), the subgroup of patients with spontaneous remission (n = 18), the subgroup of patients with progressive disease (n = 18), and healthy controls (n = 262) (numbers rounded).
0.55), 22.1% presented the homozygous genotype II (allele frequency: 0.45), and 46.3% of the patients showed the heterozygous genotype DI. In patients with spontaneous remission (n = 18), genotype DD was found in 38.9% of patients (allele frequency: 0.6), genotype II in 16.7% (allele frequency: 0.4), and the heterozygous genotype DI in 44.4% of patients. Among the patients with progressive disease (n = 18), 38.9% exhibited genotype DD (allele frequency: 0.6), 16.7% genotype II (allele frequency: 0.4), and 44.4% genotype DI. In healthy controls (n = 262), genotype DD was detected in 29.4% of the subjects (allele frequency: 0.54), genotype II was found in 21.0% (allele frequency: 0.46), and 49.6% of the subjects displayed the heterozygous genotype DI. In healthy controls as well as in patients with sarcoidosis, the distribution of genotypes was in accordance with the Hardy-Weinberg equilibrium. There were no statistically significant differences between the subgroups of sarcoid patients with regard to D/I genotype distribution.

**Distribution of AT2R1 genotypes**

The distribution of the AT2R1 A1166→C gene polymorphism was also determined by PCR (Figure 1). In the entire sarcoidosis cohort (n = 95), the homozygous genotype AA was found in 61.1% of the subjects, 11.6% disclosed the homozygous genotype CC, and 27.4% of the patients showed the heterozygous genotype AC. In patients with spontaneous remission (n = 18), genotype AA was determined in 66.7% of patients, genotype CC in 5.5%, and the heterozygous genotype AC in 27.8% of patients. Among the patients with progressive disease (n = 18), 66.7% showed genotype AA, and 11.1% genotype CC. Genotype AC was found in 22.2% of the patients. In healthy controls (n = 239), genotype AA was detected in 47.3% of the subjects, genotype CC was found in 9.2%, and 43.5% of the subjects displayed the heterozygous genotype AC. The difference in AT2R1 genotype distribution of patients with sarcoidosis and healthy controls was statistically significant (p = 0.024). In healthy controls, the distribution of genotypes was in accordance with the Hardy-Weinberg equilibrium. In male patients with sarcoidosis, however, there was a noticeable though not statistically significant accumulation of patients with the homozygous genotypes AA and CC: Genotype AA occurred in 64.8% of male sarcoid patients (expected: 56.3%), genotype CC was found in 14.8% (expected: 6.3%). Thus, the distribution of A1166→C genotypes in the entire sarcoidosis cohort was not in accordance with the Hardy-Weinberg-proportion. There were no statistically significant differences between the subgroups of sarcoid patients with regard to the A1166→C genotype distribution.

**Serum ACE levels with regard to AT2R1 and ACE genotype**

In 237 healthy controls and 77 patients with sarcoidosis without steroid therapy or ACE-blockers at the time of investigation, serum ACE levels were determined by photometric tests. As depicted in Figure 2 (a) and (b), the AT2R1 A1166→C genotype modified the influence of the D/I gene polymor-
phism on serum ACE-levels. Carrying the D allele of the ACE polymorphism resulted in higher serum ACE levels, whereas the I allele favored lower ACE levels. The A allele of the AT2R1 polymorphism supported the influence of the ACE D/I-polymorphism, whereas the C allele reduced its impact. This effect was found in healthy controls (Figure 2 (a)) as well as in patients with sarcoidosis (Figure 2 (b)).

Distribution of ACE and AT2R1 combinations

The distribution of the combined occurrence of ACE D/I and AT2R1 A1166→C was analyzed in 95 patients with sarcoidosis and 264 healthy controls. As shown in Figure 3, the combination DI*AC was significantly more common in healthy controls than in patients with sarcoidosis (Odds-Ratio 2.75; 95 % confidence interval 1.32-5.75).

Discussion

In this study, no difference was found in the distribution of ACE D/I genotypes of patients with sarcoidosis and healthy controls. Also, there was no association of ACE D/I genotypes and disease progression in sarcoidosis. The distribution of ACE D/I genotypes was in accordance with the Hardy-Weinberg equilibrium in patients with sarcoidosis and healthy controls.

Concerning the ACE D/I genotype distribution, these results are in line with studies analyzing populations in Italy, Japan, the UK, and Czechia (4-6). Also, a study in a Japanese population did not observe any influence of the ACE D/I genotypes on disease progression of sarcoidosis as determined by chest radiographs (6). Similar findings have been reported on two different populations from the UK and Czechia, where no association of ACE D/I genotype distribution and sarcoidosis disease progression, fibrosis or pulmonary disease severity was found (5).

On the other hand evidence was provided for the D allele being a potential risk factor in Japanese women with sarcoidosis (7), and in an African-American population (8). In the last mentioned study, the I allele seemed to promote disease progression (8), whereas in a Finish population, the D allele was associated with a poor prognosis (10).

Among other reasons, ethnic differences in the study populations might be the basis for these controversies. As confirmed by our data, several studies suggest that in Caucasians the frequency of the D allele is slightly higher than that of the I allele (3, 4, 10, 22). In the Japanese population, however, the I allele is predominant (6, 7). Therefore, a general statement on the role of the ACE D/I gene polymorphism in sarcoidosis might not be possible. For a definite answer, larger cohorts have to be studied by genotype-phenotype-correlation.

A role of the AT2R1 polymorphism has previously been reported in cardiovascular disease, hypertension, and arteriosclerosis (14, 23, 24). The identification of the AT2R1 A1166→C gene polymorphism led to the hypothesis of a link between this polymorphism and the incidence of sarcoidosis or sarcoidosis disease progression (12). In fact, the amount of angiotensin receptor gene expression in macrophages obtained from bronchoalveolar lavage fluid was reported to correlate with disease activity in patients with pulmonary sarcoidosis (25).

In our study, we found an increase of the homozygous AT2R1 genotypes CC and AA among male patients with sarcoidosis. These data suggest that the homozygous genotypes CC and AA might increase the risk for sarcoidosis in male subjects. This is in contrast to findings by Takemoto et al. who did...
not record any association of sarcoidosis and the AT2R1 genotypes (12).

In our study, no association was found between the ACE D/I polymorphism or the AT2R1 A1166→C gene polymorphism and sarcoidosis disease progression. We conclude that neither the ACE D/I polymorphism nor the AT2R1 A1166→C gene polymorphism plays a role in sarcoidosis progression. However, our data indicate that the homozygous AT2R1 genotypes CC and AA might increase the risk for sarcoidosis in male subjects. Similarly, it has been shown for the CC genotype to increase the risk for myocardial infarction in male patients in Norway (23). The AT2R1 A1166→C gene polymorphism modulated the effect of the ACE D/I polymorphism on serum ACE levels with the A allele promoting its impact and the C allele reducing it. Moreover, our data suggest that the co-incidence of DI and AC genotypes might be protective for sarcoidosis. Next to the ACE serum level the AT2R1 gene polymorphism might be a factor influencing the sarcoid phenotype.

Given the high variability of serum ACE levels in patients with sarcoidosis as well as healthy controls, the AT2R1 gene polymorphism has been discussed as a possible factor in the regulation of serum ACE levels in addition to the ACE gene D/I polymorphism. In patients with sarcoidosis, but not in healthy controls, Takemoto et al. described higher serum ACE levels in individuals carrying the C allele than in those having the A allele of the receptor gene (12). This is in contrast to the results of our study. We did not find a correlation of the serum ACE levels and the AT2R1 genotypes, neither in healthy control persons nor in patients with sarcoidosis. Rather, the AT2R1 genotype seemed to modulate the influence of the ACE D/I polymorphism in a way that the A allele increased the impact of the ACE D/I-polymorphism, whereas the C allele reduced it. A similar synergistic effect has been demonstrated for myocardial infarction (14-16), giving rise to the hypothesis that some allele combinations might promote different clinical outcomes.

As the use of genotype-specific normal values for the serum ACE levels has been suggested by several authors (3, 6, 7, 19), the AT2R1 A1166→C gene polymorphism could be an additional factor influencing the interpretation of serum ACE levels in the follow-up of patients with sarcoidosis.

To our knowledge, this is the first study analyzing the distribution of ACE D/I and AT2R1 A1166→C co-incidences in sarcoidosis and healthy controls. Our data indicate that the combination DI*AC is significantly less frequent in patients with sarcoidosis, suggesting a protective role of both polymorphisms against sarcoidosis. This result is surprising, as both genes are encoded on different chromosomes (ACE: chromosome 17, AT2R1: chromosome 3).

From our data, we conclude that neither the ACE D/I nor the AT2R1 A1166→C polymorphism exerts a direct role in sarcoidosis disease progression. Most interestingly, in males the homozygous AT2R1 genotypes CC and AA increase the risk for sarcoidosis but the co-incidence of the heterozygous genotypes DI and AC exerts a protective effect.

References