

ANGIOTENSIN II RECEPTOR TYPE 1 1166 A/C AND ANGIOTENSIN CONVERTING ENZYME I/D GENE POLYMORPHISMS IN A DUTCH SARCOIDOSIS COHORT

A. Kruit¹, H.J.T. Ruwen¹, J.C. Grutters², J.M.M. van den Bosch²

¹Department of Clinical Chemistry, St. Antonius Hospital, Koekoekslaan 1, 3435 CM, Nieuwegein, The Netherlands; ²Centre for Interstitial Lung Diseases, St Antonius Hospital, Department of Pulmonology, Koekoekslaan 1, 3435 CM, Nieuwegein, The Netherlands

ABSTRACT. *Background:* The angiotensin II type 1 receptor (AT2R1) is the receptor for angiotensin II, a potent vasoconstrictor produced by ACE from angiotensin I. A recent study by Biller and colleagues revealed a gender-specific association between the AT2R1 1166 A/C gene polymorphism and disease susceptibility as well as a co-dependent association between AT2R1 1166 A/C and the angiotensin-converting enzyme (ACE) insertion/deletion polymorphism on ACE levels in a group of German sarcoidosis patients. *Objective:* The aim of our study was to compare our results from Dutch Caucasian sarcoidosis patients with the results of Biller et al. *Design:* Serum and DNA from 99 patients with sarcoidosis and from 327 healthy controls were included. The AT2R1 1166 A/C and ACE I/D polymorphisms and serum ACE levels were analyzed in all subjects. *Results:* No significant differences were found between the genotype distributions between the sarcoidosis patients and controls. The genotype distributions for either polymorphism between genders and between patients with progressive/chronic disease and those with acute/remission type disease were not different. The ACE D allele contributed significantly to higher ACE levels. This was true for both sarcoidosis patients and controls. There was no association between the AT2R1 1166 A/C genotype and ACE levels, nor did AT2R1 modify the ACE D/I effects on ACE levels. No significant differences were observed in co-incidence of ACE and AT2R1 genotypes between patients and controls. *Conclusion:* Our study could not confirm the findings by Biller and colleagues other than the influence of the ACE I/D polymorphism on serum ACE levels in both sarcoidosis patients and controls. (*Sarcoidosis Vasc Diffuse Lung Dis* 2010; 27: 147-152)

KEY WORDS: sarcoidosis, gene polymorphisms, angiotensin II type 1 receptor, angiotensin, converting enzyme

INTRODUCTION

Angiotensin-converting enzyme (ACE) has been the focus of many investigations on sarcoidosis,

a granulomatous disease of unknown etiology. This is mostly due to the observation that ACE is (usually) elevated in active sarcoidosis (1). For this reason, ACE is still the mainstay marker for monitoring sarcoidosis disease activity (2). Investigations on single nucleotide polymorphisms (SNPs) have hinted at or established the importance of genetic variation in relation to either biomarkers for sarcoidosis, such as ACE, or different clinical entities of sarcoidosis. The classic example is the insertion/deletion (I/D) polymorphism in the ACE1 gene. Not only is the D allele associated with elevated ACE levels in the

Received: 26 April 2010

Accepted after Revision: 7 July 2010

Correspondence: Prof. Dr. Van den Bosch
Centre for Interstitial Lung Diseases, St Antonius Hospital,
Department of Pulmonology, Koekoekslaan 1,
3435 CM, Nieuwegein, The Netherlands
Tel. +31 (0) 30-609 9111
Fax +31 (0) 30-609 2528;
E-mail: j.vandenbosch@antoniuziekenhuis.nl

blood, while the I allele is associated with lower levels (3), carriage of particular I/D genotypes has been found to influence susceptibility and disease progression, depending on race, in sarcoidosis patients (4).

The angiotensin II type 1 receptor (AT2R1) is the receptor for angiotensin II, a potent vasoconstrictor producing ACE from angiotensin I. Carriage of the less common allele of the 1166 A/C SNP in this gene has been linked to an increased risk for myocardial infarction, but only in co-existence with homozygous DD carriage of the ACE I/D variant (5). These studies suggested an interaction between ACE and AT2R1 gene variants on disease susceptibility or severity.

A recent study by Biller and colleagues investigated whether these ACE and AT2R1 gene polymorphisms would show co-dependent associations with either disease susceptibility or progression and expression of ACE in sarcoidosis patients (6).

Our own group had previously investigated a possible role for the AT2R1 1166 A/C SNP in a group of Dutch Caucasian sarcoidosis patients, but we had not found any associations with disease susceptibility or progression (data not published). Following the publication from Biller et al. we decided to reanalyze our data using their approach by seeking interactions between the ACE I/D and AT2R1 1166 A/C polymorphisms in a group of Dutch Caucasian sarcoidosis patients.

METHODS

Patients

Ninety-nine unrelated Dutch patients with sarcoidosis (55 males/44 females; age at diagnosis (37 ± 11 years), were included in this retrospective study. ACE was measured at the time of presentation after which the diagnosis for sarcoidosis was made. None of these patients were being treated for their disease at the time of diagnosis. The diagnosis was established when clinical findings were supported by histological evidence and after exclusion of other known causes of granulomatosis in accordance with the consensus of the ATS/ERS/WASOG statement on sarcoidosis (2).

Chest radiographs were classified according to the Scadding criteria and radiographic evolution

over a 4-year follow-up period (presentation, 2 and 4 years) was categorized as follows: A (normalization or improvement towards stage I) ($n = 36$), B (persistent stage II/III or progression in that direction) ($n = 28$), and C (stable stage IV or progressive towards this stage) ($n = 6$) (7). Patients who had been diagnosed with Löfgren's syndrome at presentation ($n = 30$) were considered as a distinct group with radiographic evolution not exceeding stage I. Fourteen patients in group A, 18 in group B, 5 in group C, and 5 in the Löfgren's group received treatment with corticosteroids in the follow-up period.

Controls

Venous blood samples were obtained from 327 (117 males/210 females; age 40 ± 12 years) healthy employees of the St Antonius Hospital. By completing a questionnaire, relevant background information was provided by these volunteers, which included medication and hereditary diseases. The medical ethical committee of the St. Antonius Hospital approved the study conducted and all subjects gave formal written consent.

Measurement of ACE

Serum ACE was measured in lithium heparin plasma using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland).

Genotyping

Genomic DNA of volunteers and patients was isolated from EDTA blood using the MagNA Pure LC DNA Isolation kit I (MagNA Pure; Roche Diagnostics).

The biallelic AT2R1 1166 A/C single nucleotide polymorphism was determined using sequence-specific primers (SSPs) -polymerase chain reaction (PCR). SSPs were designed for each biallelic single nucleotide polymorphism (SNP) differing only in the 3' nucleotide. Primers were designed optimally with a length of 20 base pairs (bp), salt adjusted melting temperature (T_m) of 60°C , GC content of 50% and with no secondary structures. The program oligonucleotide properties calculator (<http://www.basic.northwestern.edu/biotools/oligoc>

alc.html) was used to determine these conditions. The sequences of SNP-specific primers were (1) 5'-GCACTTCA CTACCAAATG AGCA-3' and (2) 5'-GCACTGCA CTACCAAATG AGCA-3' for the A allele specific primer, and (3) 5'-GCACTTCA CTACCAAATG AGCC-3' and (4) 5'-GCACTGCA CTACCAAATG AGCC-3' for the C allele specific primer. The reason for using two instead of one set of primers is the presence of another SNP (T/G) that is located 16 bp upstream (shown in bold). In doing so, the alternating T/G SNP is accounted for and should not compromise the specificity of the 1166 A/C primer. Each primer was used in conjunction with a consensus primer 5'-GCA GTA CCA GGT GCA AGT GT-3', to generate an amplicon of 420 base pairs.

The SNP-specific primers were 22 bp long, had a salt adjusted melting temperature and GC content of (1) 60°C/45%, (2) 62°C/50%, (3) 62°C/50%, and (4) 64°C/55%, respectively. The consensus primer was 20 bp, had a salt adjusted melting temperature of 60°C and had a GC content of 55%.

Primers forming dimers were avoided using FastPCR (<http://primerdigital.com/fastpccr.html>). Once designed, primers were checked for specificity using the basic local alignment search tool (BLAST) <http://www.ncbi.nlm.nih.gov/BLAST/> from the national centre for biotechnology information (NCBI). The specificity of both SNP-specific primer sets was 100% for all 22 base pairs, the consensus primer was 100% for all 20 bp. The most compatible alignment with sequences other than the one used as primers in our assay was a strand of 15 bp, but the fact that the PCR reaction yielded a 420 bp product as predicted, supported the sufficient specificity of the primers used. The PCR conditions were as previously described (8).

ACE I/D polymorphisms were determined by real-time PCR using fluorescent hybridization probes and a LightCycler (Roche Diagnostics) as described earlier with some slight modifications (9-11). The detection probes were the same as described by Somogyvári et al. (10).

Statistical Analysis

Statistical analysis was performed using chi-square contingency table analysis with the appropriate number of degrees of freedom (df). Fisher's

exact test was used if expected cell frequencies were lower than five. Genotype frequencies were tested for Hardy-Weinberg equilibrium. Kruskal-Wallis or Mann-Whitney tests were used to examine differences in continuous variables between groups. The statistical evaluation of our data was performed using SPSS 16 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism 5 (Graphpad Software, Inc., San Diego, CA, USA) software packages. Multivariate ANOVA was performed on log (ln) transformed data to assess the influence of health status (sarcoidosis vs. controls), age, genotype of either ACE I/D, AT2R1 1166 A/C and interactions thereof.

Statistical significance was denoted by a value of $p < 0.05$ for all tests performed.

RESULTS

Table 1 summarizes the genotype and allele frequency distributions of ACE I/D and AT2R1 1166 A/C polymorphism in sarcoidosis patients and controls. No statistically significant differences were found between the two groups and their genotype distributions (Chi-square, $p > 0.05$ for both genes). Both gene polymorphisms were in Hardy-Weinberg equilibrium in each population.

There were no differences in genotype distributions for either polymorphism between genders or between patients with progressive/chronic disease and those with acute/remission type course of disease (data not shown).

Table 1. ACE I/D and AT2R1 allele carrier and genotype frequencies in sarcoidosis patients and controls.

Polymorphism	allele & genotype	Controls (total) (n = 327)	Patients (total) (n = 99)
ACE I/D	I	0.77 (251)	0.75 (74)
	D	0.79 (258)	0.75 (74)
	II	0.21 (68)	0.25 (25)
	ID	0.56 (182)	0.50 (49)
	DD	0.24 (77)	0.25 (25)
1166 A/C	A	0.89 (290)	0.90 (89)
	C	0.52 (172)	0.48 (48)
	AA	0.47 (155)	0.52 (51)
	AC	0.42 (135)	0.38 (38)
	CC	0.11 (37)	0.10 (10)

Chi-squared test: controls vs. patients, $p > 0.05$ for both ACE I/D and AT2R1 1166 A/C allele (carriage) frequencies.

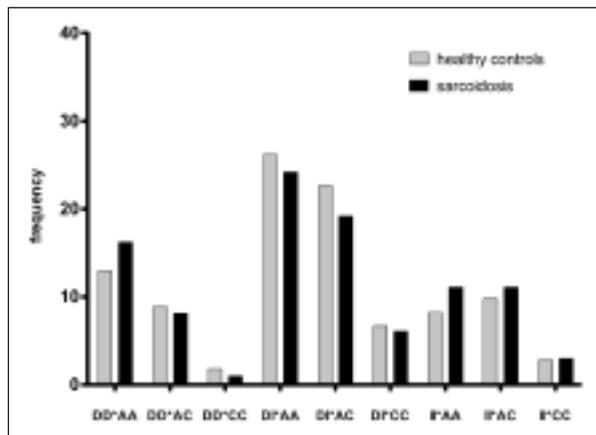


Fig. 1. Distribution of ACE and AT2R1 genotype co-incidences (%). No significant differences were found in genotype co-incidences between sarcoidosis patients (n = 99) and healthy controls (n = 327).

ACE levels were influenced by the D and I alleles, such that D carriage was associated with higher ACE levels than I carriage. This was true for both controls (median [25th-75th percentile] ACE levels (U/L): DD = 49.4 [39.5-59.5]; ID = 36.2 [29.3-44.2]; II = 25.0 [17.7-31.4]; Kruskal-Wallis, $p < 0.0001$) and sarcoidosis patients (DD = 81.1 [57.5-105.5]; ID = 66.0 [50.0-89.5]; II = 53.0 [43.0-78.5], $p = 0.01$).

There was no association between the AT2R1 1166 A/C genotype and ACE levels (data not shown). No interactions were found between the variables ACE I/D, AT2R1 1166 A/C, age, and disease state, on the ACE levels analyzed in a multivariate ANOVA.

The frequency distribution of ACE I/D and AT2R1 1166 A/C genotype co-incidences were an-

alyzed and compared between the patients and controls. The most abundant combination was found to be the DI*AA genotype combination in both controls and patients (figure 1). However, no significant differences were observed between co-occurrence of ACE and AT2R1 between patients and controls.

To test whether the 1166 A/C polymorphism could modify the ACE I/D genotype-dependent ACE levels, each 1166 A/C genotype was grouped under each ACE I/D genotype (table 2). There were no differences observed between ACE levels for any of the AT2R1 1166 A/C genotypes that were categorized in specific ACE I/D genotypes in either patients or controls.

DISCUSSION

Aside from the observed ACE genotype-dependent serum ACE levels in both healthy controls and sarcoidosis patients, ACE I/D nor AT2R1 1166 A/C genotypes did not show to have any influence on disease susceptibility or disease progression. The AT2R1 1166 A/C polymorphism, either analyzed in combination with ACE I/D or alone, had no influence on serum ACE levels.

Our study was unable to confirm any of the further observations by Biller et al. First, their study showed a significant difference in AT2R1 1166 A/C genotype distribution between patients and controls. They further stated that an increased number of AT2R1 AA and CC homozygotes were present in male sarcoidosis patients, although these results were not statistically significant. Moreover, their sarcoidosis patient population was not in keeping with the Hardy-Weinberg equilibrium for this SNP, which

Table 2. ACE I/D and AT2R1 dependent ACE levels (U/L) in sarcoidosis patients and controls.

ACE I/D	DD			ID			II		
	AA	AC	CC	AA	AC	CC	AA	AC	CC
Controls	(n=42)	(n=29)	(n=6)	(n=86)	(n=74)	(n=22)	(n=27)	(n=32)	(n=9)
Median ACE	47	53	49	35	37	36	25	23	31
Range*	(36-58)	(44-65)	(44-68)	(28-44)	(30-48)	(31-42)	(20-29)	(17-31)	(22-34)
Patients	(n=16)	(n=8)	(n=1)	(n=24)	(n=19)	(n=6)	(n=24)	(n=19)	(n=6)
Median ACE	71	90	78	68	64	63	68	64	64
Range	(57-100)	(50-133)		(54-79)	(45-92)	(54-79)	(54-79)	(45-92)	(51-86)

* range is 25th and 75th percentiles.

Kruskal-Wallis test: $p = 0.89$; every column comparison (post-test) within each ACE I/D genotype: $p > 0.05$ for either the controls or the patients.

questions the integrity of the patient selection, unless the AT2R1 A/C would strongly impact the likelihood of a patient contracting sarcoidosis. In comparing our results with those of Takemoto (12), the frequency distributions of both the ACE and AT2R1 genotypes were quite different from those in the Japanese population, although these were no different between patients and controls in each respective study group. Previous Japanese studies have shown that compared to Caucasian populations, the ACE I/D genotype distribution is different, with preponderance of the I allele in the Japanese. The observation that the AT2R1 1166 A/C frequency in Japanese is equally different from Caucasians is therefore not surprising and denotes the genetic diversity between races and the relative influence of allele carriage in disease susceptibility.

The second observation by Biller et al. was the increased number of co-incidence of ACE DI with AT2R1 1166 AC (DI*AC) in the sarcoidosis patients, suggesting a protective effect of co-inheritance of this genotype combination. Our study did not show any differences in terms of co-incidence between patients and controls. The similarity between the German and Dutch ancestry would predict a comparable genetic make-up and thus a similar frequency distribution of polymorphisms. For the German and Dutch control groups this was indeed the case, but not for the sarcoidosis groups of different nationalities, which revealed differences in AT2R1 1166 A/C genotype frequency distributions. However, seeing as sarcoidosis is a multifactorial disease, the results found by Biller et al. may very well be of significance in German sarcoidosis patients, but not in Dutch sarcoidosis patients. Another possible explanation for the different results between the German and Dutch patients may be the disease phenotypes in each cohort. For example, the German patient cohort had a much higher share of patients with stage II and III, while the Dutch had a preponderance of patients with stage I disease. Although no associations were found between radiographic staging and AT2R1 genotype distributions in either study, it may be that the limited size of both populations conceals such an association. As a result, the net genotype distributions between the Dutch and German cohort may differ slightly and thus yield different results.

Finally, the interaction between the ACE DI and AT2R1 1166 AC polymorphisms on the serum

ACE levels was not seen in our study groups. The question is whether such effect was even found in Biller's study, seeing as no statistical analysis supported their observation. Only if such observations were to be proven statistically significant, the interaction between the two genes in their genotype would suggest a sub-additive model for ACE expression by the AT2R1 1166 C allele in both healthy and disease states. Our data showed narrow (<11%) 95% CI ranges, which translates to an acceptable statistical power to detect differences between ACE levels. Consequently, differences in genotype-specific ACE levels as low as 11% would have been able to be identified.

In conclusion, our study did not find what was observed by Biller and colleagues other than the influence of the ACE I/D polymorphism on serum ACE levels in both sarcoidosis patients and healthy individuals.

ACKNOWLEDGMENTS

The authors would indebted to Jan Broess and Natalie Pot for their technical support and primer design and to Dr. Pieter Zanen for his support with the statistical analyses..

REFERENCES

- Lieberman J. Elevation of serum Angiotensin-converting-enzyme (ACE) level in sarcoidosis. *Am J Med* 1975; 59: 365-72.
- Hunninghake GW, Costabel U, Ando M, et al. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. *Sarcoidosis Vasc Diffuse Lung Dis* 1999; 16: 149-73.
- Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the Angiotensin I-Converting Enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990; 86: 1343-6.
- Medica I, Kastrin A, Maver A, Peterlin B. Role of genetic polymorphisms in ACE and TNF-alpha gene in sarcoidosis: A meta-analysis. *J Hum Genet* 2007; 52: 836-47.
- Tiret L, Bonnardeaux A, Poirier O, et al. Synergistic effects of Angiotensin-Converting Enzyme And Angiotensin-II type 1 receptor gene polymorphisms on risk of myocardial infarction. *Lancet* 1994; 344: 910-3.
- Biller H, Ruprecht B, Gaede KI, Muller-Quernheim J, Zissel G. Gene polymorphisms of ACE and the Angiotensin Receptor AT2R1 influence serum ACE levels in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2010; 26: 139-46.
- Grutters JC, Sato H, Pantelidis P, et al. Analysis of IL6 and IL1a gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2003; 20: 20-7.

8. Bunce M, O'Neill CM, Barnardo MC, et al. Phototyping: Comprehensive DNA typing for HLA-a, b, c, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (pcr-ssp). *Tissue Antigens* 1995; 46: 355-67.
9. Rigat B, Hubert C, Corvol P, Soubrier F. PCR detection of the insertion/deletion polymorphism of the human Angiotensin Converting Enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res* 1992; 20: 1433.
10. Somogyvari F, Szolnoki Z, Marki-Zay J, Fodor L. Real-time PCR assay with fluorescent hybridization probes for exact and rapid genotyping of the Angiotensin-Converting Enzyme gene insertion/deletion polymorphism. *Clin Chem* 2001; 47: 1728-9.
11. Lindpaintner K, Pfeffer MA, Kreutz R, et al. A prospective evaluation of an Angiotensin-Converting-Enzyme gene polymorphism and the risk of ischemic heart disease. *N Engl J Med* 1995; 332: 706-11.
12. Takemoto Y, Sakatani M, Takami S, et al. Association between Angiotensin II Receptor gene polymorphism and serum Angiotensin Converting Enzyme (ACE) activity in patients with sarcoidosis. *Thorax* 1998; 53: 459-62.