

Significant Association of Insertion/Deletion Polymorphism of the Angiotensin-Converting Enzyme Gene with Rheumatoid Arthritis

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ABSTRACT. *Objective.* Only 30% of the genetic contribution to rheumatoid arthritis (RA) can be attributed to HLA genes, and other non-HLA genes may play a role in RA susceptibility. Angiotensin-converting enzyme (ACE) has been reported to be involved in pathogenesis of RA, and high levels of ACE have been documented in RA synovial fluid and pleural effusions. Since plasma and tissue levels of ACE are determined at the transcriptional level, we test the hypothesis that the genotype of ACE in RA patients may be a determining factor in pathogenesis.

Methods. Sixty patients with RA were recruited and clinically characterized according to disease duration, disease severity, disease activity, and American College of Rheumatology functional classes. ACE gene I/D polymorphism genotypes were determined in patients and healthy controls, using polymerase chain reaction.

Results. We found a significant overrepresentation of the DD genotype and the D allele in patients with RA; and we found that men with RA exhibited a higher frequency of the DD genotype and D allele compared to male controls. By logistic regression analysis the DD genotype confers a relative risk for development of RA of 3.

Conclusion. Our study found an association between ACE deletion polymorphism and RA. (First Release Nov 1 2007; J Rheumatol 2007;34:2395–9)

Key Indexing Terms:

ANGIOTENSIN-CONVERTING ENZYME KUWAIT RHEUMATOID ARTHRITIS
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The renaissance in the study of the genetic susceptibility to rheumatoid arthritis (RA), the commonest chronic inflammatory polyarthritis seen in rheumatology practice, is important in view of its diagnostic and therapeutic potentials. Only 30% of the genetic contribution to RA can be attributed to HLA genes, and it is suggested that other non-HLA genes play a role in RA susceptibility¹. Patients with RA are believed to share some common pathogenic mechanisms that involve inflammation and elaboration of reactive oxygen species^{2,3}. Recently, angiotensin-converting enzyme (ACE), a key regulator in inflammatory signal transduction pathways, has been

reported to be involved in pathogenesis of RA, and high levels of ACE have been documented in RA synovial fluid^{4,5} and RA pleural effusions⁶. An insertion (I)/deletion (D) polymorphism of a 287 base-pair fragment in intron 16 of the ACE gene has been found to account for 47% of the total variance of plasma ACE activity⁷. It has been confirmed that the serum ACE levels correspond to the order II < ID < DD genotypes in healthy individuals^{8,9}. Since plasma and tissue levels of ACE are determined at the transcriptional level, we hypothesized that the genotype of ACE gene in patients with RA may be a determining factor in the onset and pathogenesis of this inflammatory disease.

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MATERIALS AND METHODS

Patient recruitment and evaluation. Sixty consecutive patients with RA attending the Rheumatology Clinic at Mubarak Al-Kabeer Hospital, Kuwait, were recruited and ACE gene I/D polymorphism genotypes were determined in them. All patients fulfilled the American College of Rheumatology (ACR) classification criteria for RA¹⁰ and gave informed consent for the study. A complete clinical evaluation was done for all patients. Standardized joint counts and patient ratings of general health on a 100 mm visual analog scale were recorded. Data were entered into a predesigned proforma. The 4-variable Disease Activity Score 28 (DAS28-4)¹¹ was computed using the formula: $0.56 \times \sqrt{(28\text{TJC})} + 0.28 \times \sqrt{(28\text{SJC})} + 0.70 \times \ln(\text{ESR}) + 0.014 \times \text{GH}$, where TJC = tender joint count, SJC = swollen joint count, ESR = erythrocyte sedimentation rate, and GH = general health. A cutoff point of < 2.6 was used for

the DAS28-4 to define remission, corresponding with fulfillment of the modified American Rheumatism Association criteria for remission^{12,13}.

The patients were clinically characterized on the basis of disease duration into having early (< 3 mo), established (> 3 mo to up to 2 yrs), or late (> 2 yrs) disease^{14,15}; on the basis of maximum number of joints involved at any stage into mild (3–6 joints), moderate (> 6 but < 20 joints), or severe (> 20 joints) disease^{15,16}; on the basis of assessed disability into American College of Rheumatology (ACR) functional class I, II, III, or IV; and on the basis of DAS28-4 as being in remission (< 2.6), or being active (> 2.6)¹⁷.

Treatment strategy for patients with RA followed guidelines published recently¹⁸. All patients were treated with disease modifying antirheumatic drugs (DMARD) and/or biologicals, and whenever appropriate nonsteroidal antiinflammatory drugs (NSAID) and/or corticosteroids were added.

Thirty-five healthy controls were also enrolled in the study.

Genotyping. Blood samples were collected from patients and controls. Total genomic DNA was isolated by a standard procedure¹⁹. ACE genotypes were determined by polymerase chain reaction (PCR) using primers and conditions described previously⁷. Reactions were performed with 10 pmol of each primer: sense oligo: 5' CTG GAG ACC ACT CCC ATC CTT TCT 3' and anti-sense oligo: 5' GAT GTG GCC ATC ACA TTC GTC AGA T 3' in a final volume of 50 μ l, containing 3 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.1 mg/ml gelatin, 0.5 mM of each dNTP (Cetus), 2.5 u AmpliTaq DNA polymerase (Cetus). DNA was amplified for 30 cycles with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min using a Perkin-Elmer thermal cycler. 0.6% dimethylsulfoxide is routinely added to the PCR mix in order to improve the amplification of the I allele and avoid mistyping it as a D allele. PCR products were analyzed on 2% agarose gels after staining with ethidium bromide. In the absence of the 287 bp insertion in intron 16 of the ACE gene, this PCR method resulted in a 190 bp product (D allele) and in the presence of insertion, produced a 490 bp product (I allele). In heterozygous samples, 2 bands (490 and 190 bp) were detected along with a third fragment of intermediate size, which corresponds to a heteroduplex DNA fragment²⁰. In all PCR analyses, appropriate positive and negative controls were included.

Statistical methods. Statistical analysis was performed using SPSS v 12.0 (SPSS Inc., Chicago, IL, USA) using the level of $p \leq 0.05$ as statistically significant. Chi-square test and Fisher's exact were used to assess the association between 2 qualitative variables. Kruskal-Wallis test was used to correlate gene polymorphism and age at onset in patients with RA. Logistic regression was used to estimate the risk of factors in RA after controlling confounding between them. The variables sex and ethnicity were included in the model to control for confounding. The adjusted odds ratios (OR) and their 95% confidence intervals (CI) for associated factors were computed from the coefficients of the conditional logistic regression model. The allele and genotype frequencies followed normal Hardy-Weinberg distribution in our population.

RESULTS

Genotype and allele frequencies subgrouped by sex and ethnicity are presented in Tables 1 and 2.

First, the ACE genotype frequencies were delineated. A significant difference was found in ACE I/D genotype frequencies between patients and controls ($p = 0.014$), with an overrepresentation of the DD genotype in patients compared to controls. In addition, when a comparison was made between the demographic subgroups of patients with RA, it was noted that sex correlated significantly with genotypic frequencies. Importantly, male patients with RA exhibited a higher frequency of the DD genotype compared to male controls. In contrast, when Arab patients were compared to non-Arabs, ethnicity did not translate into any significant differences in ACE genotypes.

Next, the frequencies of ACE alleles were delineated. A significant difference was found in frequencies of ACE I/D alleles between patients and controls, with RA patients having a higher representation of D and lower representation of I alleles compared to controls. When comparing frequencies of ACE I/D alleles between each subgroup of patients and controls, a higher frequency of D allele was found in male patients with RA compared to male controls, and Arab patients showed a similar higher frequency of D allele compared to Arab controls. No such difference could be found in non-Arabs.

Next, when a comparison was made between the subgroups of patients with RA as per disease variables (Table 3), a significant correlation was observed between ACR functional class of patients with RA and ACE genotypes ($p = 0.035$). Additionally, although the frequency of D allele was higher in patients with severe disease (22.5% vs 12.9%) compared to controls, these figures did not reach statistical significance. However, no correlation was observed for DAS28 scores, TJC, SJC, ESR, disease duration, or requirement for DMARD with regard to frequency of ACE genotypes or alleles (data not shown).

Finally, logistic regression analysis was used to adjust confounding between demographic factors. Table 4 presents the

Table 1. Angiotensin-converting enzyme (ACE) allele and genotype frequencies in patients with RA and healthy controls.

Variable	Male		p	Female		p	All		p
	Controls, n = 14 n (%)	Patients, n = 19 n (%)		Controls, n = 21 n (%)	Patients, n = 41 n (%)		Controls, n = 35 n (%)	Patients, n = 60 n (%)	
ACE genotype frequency			0.031 ^{b*}			0.085 ^b			0.014 ^{a*}
II	8 (57.1)	6 (31.6)		2 (9.5)	4 (9.7)		10 (28.6)	10 (16.7)	
ID	4 (28.6)	2 (10.5)		9 (42.9)	9 (22.0)		13 (37.1)	11 (18.3)	
DD	2 (14.3)	11 (57.9)		10 (47.6)	28 (68.3)		12 (34.3)	39 (35.0)	
ACE allele frequency			0.006 ^{a*}			0.208			0.003 ^{a*}
I	20 (71.4)	14 (36.8)		13 (31.0)	17 (20.7)		33 (47.1)	31 (26.8)	
D	8 (28.6)	24 (63.2)		29 (69.0)	65 (79.3)		37 (52.9)	89 (74.2)	

p values calculated by ^a chi-square test and ^b Fisher's exact test. * Values are significant.

Table 2. Angiotensin-converting enzyme (ACE) allele and genotype frequencies in patients with RA and control subjects according to ethnicity.

Variable	Arabs		p	Non-Arabs		p
	Controls, n = 20 n (%)	Patients, n = 36 n (%)		Controls, n = 15 n (%)	Patients, n = 24 n (%)	
ACE			0.082 ^b			0.158 ^a
II	4 (20.0)	3 (8.3)		6 (40.0)	7 (29.2)	
ID	7 (35.0)	6 (16.7)		6 (40.0)	5 (20.8)	
DD	9 (45.0)	27 (75.0)		3 (20.0)	12 (50.0)	
Allele frequency			0.014 ^{a*}			0.079 ^a
I	15 (37.5)	12 (16.7)		18 (23.1)	19 (24.4)	
D	25 (62.5)	60 (83.3)		12 (15.4)	29 (37.2)	

p values calculated by ^a chi-square test and ^b Fisher's exact test. * Values are significant.

Table 3. Angiotensin-converting enzyme (ACE) allele and genotype frequencies in 60 patients with RA as per disease variables.

	Genotype			p	Allele Frequency		p
	DD, n = 39 n (%)	ID, n = 11 n (%)	II, n = 10 n (%)		D, n = 89 n (%)	I, n = 31 n (%)	
Sex				0.097 ^b			0.061 ^a
Male	11 (28.2)	2 (18.2)	6 (60.0)		24 (27.0)	14 (45.2)	
Female	28 (71.8)	9 (81.8)	4 (40.0)		65 (73.0)	17 (54.8)	
Nationality				0.076 ^b			0.005 ^{a*}
Arab	34 (87.2)	11 (100)	8 (80.0)		60 (67.4)	12 (38.7)	
Non-Arab	5 (12.8)	0 (0.0)	2 (20.0)		29 (32.6)	19 (61.3)	
Age at onset, yrs				0.694 ^b			0.390 ^a
≤ 40	24 (61.5)	7 (63.6)	6 (60.0)		38 (42.7)	16 (51.6)	
> 40	15 (38.5)	4 (36.4)	4 (40.0)		51 (57.3)	15 (48.4)	
Functional class				0.035 ^{b*}			0.285 ^a
Daily activity, work, recreation	13 (34.2)	7 (63.6)	5 (50.0)		33 (37.9)	17 (54.8)	
Daily activity, work	17 (44.7)	0 (0.0)	4 (40.0)		34 (39.1)	8 (25.8)	
Daily activity	8 (21.1)	4 (36.4)	1 (10.0)		20 (23.0)	6 (19.4)	

p values generated by ^a chi-square test, and ^b Fisher's exact test. * Values are significant.

risk of RA according to ACE genotype, using logistic regression. Step 1 included patients with RA and controls as the dependent variables, with genotype as per sex and nationality being independent variables. A person with genotype DD was found to be 3.25 times more at risk to develop RA than a person with II genotype (unadjusted OR 3.25, 95% CI 1.09–9.66, $p = 0.034$). ID genotype gave no significant risk. In step 2, when confounded by the sex variable, the risk conferred by DD genotype was reduced to 3.01. In step 3, when confounded by nationality, risk of DD increased significantly to 3.55. In step 4, when confounded by both sex and ethnicity, the relative risk for RA conferred by the DD genotype was 3.21 times that of the II genotype (95% CI 0.98–10.55, $p = 0.055$).

DISCUSSION

The genetic basis for rheumatologic diseases continues to be a fruitful area of research, with its potential implications in diagnosis, treatment, and disease outcome. The contribution of human leukocyte antigen (HLA) DR genes, particularly the

HLA-DRB1 gene, to genetic predisposition of RA was the first described, and remains as the best characterized genetic risk factor contributing to RA²¹. The associations between RA and HLA-DR genes are, however, incomplete. It has recently been shown that 2 regions in the MHC, class II (DR-β1) and class III (D6S273, HSP70, Bat2, TNFα), more completely define the risk for development of RA²². The above notwithstanding, it has been estimated that only 30% of the genetic contribution to RA can be attributed to HLA genes and it is suggested that other non-HLA genes play a role in RA susceptibility¹.

To view the genetic susceptibility to RA from another standpoint, patients with RA are believed to share some common pathogenic mechanisms that involve inflammation and elaboration of reactive oxygen species^{2,3}.

Recently, ACE, a key regulator in inflammatory signal transduction pathways, has been reported to be involved in pathogenesis of RA. Elevated synovial fluid ACE levels have been demonstrated, indicating that locally generated ACE could contribute to joint destruction in RA^{4,5}. High levels of

Table 4. Risk of ACE gene polymorphism on RA estimated by logistic regression analysis.

Variable	Odds Ratio	95% CI	p
Step 1			
ACE			
II (reference)	1.00		
ID	0.85	0.26–2.78	0.783
DD	3.25	1.09–9.66	0.034
Step 2			
Sex			
Male	0.84	0.31–2.24	0.727
Female (reference)	1.00		
ACE			
II (reference)	1.00		
ID	0.78	0.22–2.79	0.704
DD	3.01	0.94–9.69	0.065
Step 3			
Nationality			
Arab	0.80	0.32–2.04	0.643
Non-Arab (reference)	1.00		
ACE			
II (reference)	1.00		
ID	0.88	0.27–2.94	0.838
DD	3.53	1.12–11.10	0.031
Step 4			
Sex			
Male	0.73	0.25–2.13	0.567
Female (reference)	1.00		
Nationality			
Arab	0.72	0.26–1.98	0.519
Non-Arab (reference)	1.00		
ACE			
II (reference)	1.00		
ID	0.78	0.22–2.80	0.707
DD	3.21	0.98–10.55	0.055

Step 1: unadjusted; Step 2: adjusted for sex; Step 3: adjusted for nationality; Step 4: adjusted for sex and nationality.

ACE have also been reported in RA pleural effusions⁶. The ACE gene, located on chromosome 17, plays a key role in the renin-angiotensin and kallikrein-kinin systems. ACE converts angiotensin I to angiotensin II (AT II) by release of the terminal His-Leu, resulting in increased vasoconstrictor activity, and also causes inactivation of bradykinin, a potent vasodilator. It is being increasingly realized that, beyond its hemodynamic effects, AT II has a role as a growth factor and as a proinflammatory modulator, participating in the key events of the inflammatory response^{23,24}.

The ACE gene locus is the major locus that determines serum ACE concentration. An I/D polymorphism of a 287 base-pair fragment in intron 16 of the human ACE gene has been found to account for 47% of the total variance of plasma ACE activity⁷. Plasma ACE activity is increased by 20%–40% for ID and 50%–70% for DD versus II genotypes ($p < 0.001$)²⁵. It has been confirmed that serum ACE levels correspond to the order II < ID < DD in healthy individuals^{8,9}.

Since plasma and tissue levels of ACE are determined at the transcriptional level, we decided to investigate the hypoth-

esis that the genotype of ACE in patients with RA may contribute to the onset and pathogenesis of this inflammatory disease. In our study, we detected a significant overrepresentation of the DD genotype and the D allele in patients with RA when compared to controls. Additionally, we found that sex correlates significantly with genotypic and allele frequencies, with male RA patients exhibiting a higher frequency of the DD genotype and D allele compared to male controls. Further, Arab patients show a higher frequency of D allele compared to Arab controls. No such differences exist in females and in non-Arabs. By logistic regression analysis, we found that the DD genotype confers a relative risk for development of RA of around 3. Our results also suggest a possible influence of the ACE gene on RA disease severity and functional classes.

ACE gene I/D polymorphisms are also implicated in the pathogenesis of a variety of cardiovascular disorders including myocardial infarction, left ventricular hypertrophy, and hypertension, and the ACE gene has been recognized as a top candidate gene for cardiovascular research²⁶. A large number of studies have shown a positive association between the DD genotype and an increased risk of myocardial infarction²⁷. A higher incidence of hypertension *per se*²⁸ and of left ventricular hypertrophy in patients with hypertension²⁹ has also been shown with this genotype, and also an increased risk of coronary heart disease and cardiovascular mortality in smokers³⁰. Indeed it has been reported that the D allele behaves as a marker of atherosclerotic cardiovascular complications³¹. Thus, taken together, the available evidence supports the notion that the DD genotype adversely influences specific cardiovascular diseases. Given the association between RA and cardiovascular diseases³²⁻³⁴, is it possible that the same pathway (ACE pathway) and the same genotype (DD) are involved in both these seemingly diverse conditions? This could enable this polymorphism and its association with RA, on the one hand, and cardiovascular diseases, on the other, to be placed in a broader perspective.

A significant association of the I allele has been demonstrated with the 3 clinical subclasses of juvenile idiopathic arthritis (JIA), with the highest association being observed in systemic JIA³⁵. Thus, while the I allele predisposes to development of juvenile arthritis, our data suggest that the D allele increases the propensity to develop adult RA. These contrasting findings may be explained if one considers that these 2 disorders follow somewhat different pathways at the cellular and molecular level. Thus, a variety of recent data suggest that systemic-onset JIA, and possibly other JIA subtypes, are “autoinflammatory” diseases^{36,37} in contrast to adult seropositive RA, which is an “autoimmune-inflammatory disease.” It is therefore plausible that these 2 diseases follow diverse inflammatory pathways.

Our results suggest that the ACE gene contributes to the heritability of RA susceptibility. Our study is limited in its power, but being the first in this field, could provide impetus for larger and more powerful studies in other populations/eth-

nic groups to explore the relationship between ACE gene polymorphisms and susceptibility to RA.

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