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jraas

Journal of the Renin-Angiotensin-Aldosterone System
12(4) 549–556

DOI: 10.1177/1470320311399605
jra.sagepub.com
SAGE

Polymorphisms of the angiotensin-converting enzyme and angiotensinogen gene in patients with atrial fibrillation

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Abstract

Activation of the renin–angiotensin system (RAS) is associated with atrial fibrillation (AF). The aim of this study was to investigate the relation between AF and polymorphisms in RAS. One hundred and fifty patients with AF, 100 patients with no documented episode of AF and 100 healthy subjects were consecutively recruited into the study. The angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism, and the M235T, A-20C, and G-6A polymorphisms of the angiotensinogen gene were genotyped. Patients with AF had significantly lower frequency of II genotype of ACE I/D and higher frequency of angiotensinogen M235T polymorphism T allele and TT genotype and G-6A polymorphism G allele and GG genotype compared with the controls. AF patients had significantly larger left atrium, higher left ventricular mass index (LVMI) and higher frequency of significant valvular pathology. ACE I/D polymorphism II genotype, angiotensinogen M235T polymorphism TT genotype and G allele and GG genotype of angiotensinogen G-6A polymorphism were still independently associated with AF when adjusted for left atrium, LVMI and presence of significant valvular pathology. Genetic predisposition might be underlying the prevalence of acquired AF. Patients with a specific genetic variation in the RAS genes may be more liable to develop AF.

Keywords

Angiotensin-converting enzyme, angiotensinogen, atrial fibrillation, genetic susceptibility, polymorphism, renin–angiotensin system.

Introduction

Atrial fibrillation (AF) is the most commonly observed arrhythmia in clinical practice and associated with increased cardiovascular morbidity and mortality.[1-3] Atrial fibrosis and loss of atrial muscle mass are the most important pathological changes observed in patients with AF.[4,5] Valvular heart disease, hypertension and atherosclerosis may precipitate AF by dilating the atria, which in turn triggers atrial fibrosis.[6]

The renin–angiotensin system (RAS) has been shown to be involved in many cardiovascular diseases, including myocardial fibrosis and hypertrophy in hypertensive heart disease,[7] congestive heart failure,[8] myocardial infarction,[9] and cardiomyopathy.[10] Increased angiotensin-converting enzyme (ACE) expression in the atrial tissue of patients with AF has suggested the involvement of the RAS in AF.[11,12] RAS is activated by atrial wall tension and increases angiotensin II level.[13] Angiotensin II activates the mitogen-activated protein kinase pathway,[11,14] and induces the proliferation of fibroblasts, extracellular matrix protein accumulation and hypertrophy of cardiomyocytes,[11,15] conduction heterogeneity,[14] and decreased atrial effective refractory period,[16] and provides the substrates for the development of AF. Thus angiotensin II might be involved

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in the mechanism of atrial structural and electrical remodellings. Inhibition of the cardiac RAS by ACE inhibitors or angiotensin receptor antagonists might affect the pathophysiological substrate of AF and offer a new therapeutic approach for AF.[17-19]

Genetic variations of RAS affect plasma and tissue RAS activity, and ACE and angiotensin II levels. A polymorphism consisting of an insertion/deletion (I/D) of a 287-bp fragment has been identified in the intron 16 of the gene encoding for the ACE, and the ACE D allele has been reported to be associated with increased plasma and cellular ACE levels.[20-22] The ACE DD genotype is associated with higher plasma levels of the enzyme, the II genotype with lower ACE levels, and the ID genotype with intermediate levels.[20] The ACE I/D is an appealing polymorphism in relation to susceptibility to cardiovascular diseases, including hypertensive heart disease,[23] congestive heart failure,[24] coronary artery disease,[25] and cardiomyopathy.[26] In addition, the ACE DD genotype has been shown as a predisposing factor for AF.[26-28] Darbar *et al.*[29] demonstrated a pharmacogenetic interaction between the ACE I/D polymorphism and efficacy of antiarrhythmic drug therapy in patients with lone AF. Polymorphisms in the angiotensinogen gene might also be associated with AF as they may cause higher angiotensinogen gene transcription activity and a higher tissue angiotensin II concentration in the atrium under the stimulation of high atrial pressure.[30,31]

The aim of this study was to investigate the association between AF and polymorphisms of RAS (particularly, ACE gene I/D polymorphism and the M235T, A-20C, and G-6A polymorphisms of the angiotensinogen gene) in a Turkish population.

Materials and methods

The investigation complies with the principles outlined in the Declaration of Helsinki. The study was approved by the local ethics committee and all the participants gave written informed consent before participating in the study.

One hundred and fifty patients with chronic AF were consecutively included into the study as the AF group. One hundred consecutive patients with no documented episode of AF or any other rhythm problem and 100 healthy subjects were included as control group 1 and control group 2, respectively. Patients with recent (< 3 months) myocardial infarction, pericarditis, myocarditis, hyperthyroidism, malignancy, pulmonary embolism or acute pulmonary diseases (pneumonia, exacerbation of chronic obstructive pulmonary disease) and postoperative AF were excluded. None of the AF was familial.

The presence of AF was documented by serial ECG and/or ambulatory ECG monitoring. Patients with palpitations without ECG documentation were excluded from both the patient and control groups. A questionnaire on smoking habits, medical history of CAD, diabetes, hyperlipidemia, hypertension, and renal failure was filled out by each participant. All subjects underwent physical examination. Weight and height were measured to determine body mass index (BMI). Blood samples were obtained to determine genotypes. Transthoracic echocardiography was performed to measure left atrial and left ventricular dimensions and left ventricular ejection fraction, and to detect significant valvular disease (defined as moderate or severe valvular regurgitation or stenosis with any severity). Left ventricular mass (LVM) was calculated with echocardiographic parameters and the Devereux formula.[32]

Genotyping

Peripheral blood samples from all of the patients and controls were collected into EDTA-coated tubes. Genomic DNA was extracted from peripheral blood leukocytes with DNA isolation kit (Roche Diagnostic, High Pure DNA Isolation Kit). DNA fragments were amplified by polymerase chain reaction (PCR). The stepdown PCR method was performed to identify ACE gene I/D polymorphism according to the previously published protocols.[33] The I/D polymorphism of the ACE gene was assessed by detecting the presence (allele I, insertion) or absence (allele D, deletion) of a 287-bp sequence in the intron 16 of the ACE gene in the chromosome 17. A set of primers was designed to encompass the polymorphic region in the intron 16 of the ACE gene (sense primer 5' CTGGAGACCACTCCCATCCTTTCT 3' and antisense primer 5' GATGTGGCCATCACATTCGTCAGAT 3'). DNA was amplified for 35 cycles, each cycle was composed of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR products (190 bp D allele and 490 bp I allele) were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light.

The possibility of mistyping ID heterozygotes as DD homozygotes due to the preferential amplification of the smaller D allele was addressed.[34] To verify the DD allele, each sample found to be DD was subjected to a second, independent PCR with a primer pair (hace5a and hace5c) that permits amplification only in the presence of the I allele, but not the D allele. This test was performed using the method described by Lindpaintner *et al.*[35]

For genotyping of angiotensinogen gene polymorphisms, we used mini-PCR direct sequencing as described previously.[36] Genomic DNA was extracted by a non-enzymatic method. DNA fragments were amplified by PCR. Forward and reverse primers were selected from the genomic sequence of angiotensinogen. The forward primer sequence from +921 to +940 in exon 2 of the angiotensinogen gene was 5'-GAT GCG CAC AAG GTC CTG TC-3'; the reverse primer from +1255 to +1274 was 5'-GCC AGC AGA GAG GTT TGC CT-3'. The PCR mixture consisted

of 0.5 µg DNA, 25 pmol of each primer, 0.15 nmol/L dNTP and 1 U Taq polymerase in a final volume of 50 µl. The reaction condition was achieved first by denaturation for 3 min, and then repeating the following cycle: denaturation at 95°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min. This cycle was repeated 30 times with a final extension for 10 min. The 394 bp PCR product was resolved on a 2% ethidium bromide stained gel and purified by centrifugation through a paper slurry for sequencing.

The operators who performed the genotype analysis were unaware of the patients' characteristics.

Statistical analysis

The statistical analysis was performed with a commercially available statistical analysis programme (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). Categorical variables were expressed as frequency (percentage) while continuous variables were expressed as mean ± standard deviation. Pearson chi-square (χ^2) test was used to compare groups regarding the categorical variables. Continuous variables were compared with Student's *t*-test (while comparing parametric variables between two groups), Mann–Whitney *U*-test (while comparing non-parametric variables between two groups), ANOVA test (while comparing parametric variables between three groups), and Kruskal–Wallis test (while comparing non-parametric variables between three groups). A logistic regression analysis was modelled to determine the independent predictors of AF in this cohort of patients. p values < 0.05 were interpreted as statistically significant. Genetic variation was distributed according to the Hardy–Weinberg equilibrium (deviation from the Hardy–Weinberg equilibrium was assessed using (χ^2) statistics).

Results

Baseline characteristics of the study subjects are presented in table 1. There were no significant differences in age, gender or BMI among the three study groups. Patients with AF had significantly higher frequency of significant valvular pathology compared with patients without AF (< 0.001). Echocardiographic measures of the study subjects are presented in table 2. Patients with AF had significantly larger left atrium and higher left ventricular mass index (LVMI) than the control groups.

Table 1. Baseline characteristics of the study subjects

	AF (+) patients (n = 150)	Control group 1 AF (-) patients (n = 100)	Control group 2 Healthy controls (n = 100)	p
Gender (F/M)	65/85	37/63	41/59	0.607
Age (year)	67.9 ± 9.8	65.8 ± 12.4	68.2 ± 10.2	0.213
BMI (kg/m^2)	27.85 ± 5.22	27.59 ± 4.15	28.01 ± 4.42	0.692
Smokers (n, %)	55 (36.7%)	39 (39.0%)	38 (38.0%)	0.931
Valvular pathology (n, %)	120 (80.0%)	33 (33.0%)	–	**< 0.001**
HT (n, %)	113 (75.3%)	84 (84.0%)	–	0.100
DM (n, %)	48 (32.0%)	43 (43.0%)	–	0.077
CAD (n, %)	50 (33.3%)	45 (45.0%)	–	0.063
HL (n, %)	67 (44.7%)	57 (57.0%)	–	0.056
CRF (n, %)	32 (21.3%)	28 (28.0%)	–	0.227
CVD (n, %)	38 (25.3%)	13 (13.0%)	–	**0.018**

BMI: body mass index, HT: hypertension, DM: diabetes mellitus, CAD: coronary artery disease, HL: hyperlipidemia, CRF: chronic renal failure, CVD: cerebrovascular disease.

Table 2. Echocardiographic measures of study subjects

	AF (+) patients (n = 150)	Control group 1 AF (-) patients (n = 100)	Control group 2 Healthy controls (n = 100)	p
LA (cm)	5.01 ± 0.86	4.04 ± 0.52	3.85 ± 0.22	**< 0.001**
LVED (cm)	5.11 ± 0.82	4.96 ± 0.73	4.88 ± 0.57	0.137
LVES (cm)	3.48 ± 1.10	3.30 ± 0.99	3.14 ± 0.78	0.290
IVS (cm)	1.22 ± 0.16	1.18 ± 0.14	1.02 ± 0.10	0.053
PW (cm)	1.14 ± 0.15	1.09 ± 0.12	1.01 ± 0.09	0.066
LVEF (%)	53 ± 12	53 ± 13	61 ± 6	0.089
LVMI (g/m^2)	164.3 ± 50.3	147.9 ± 39.2	115.6 ± 19.7	**< 0.001**

LA: left atrial diameter, LVED: left ventricular end-diastolic diameter, LVES: left ventricular end-sistolic diameter, IVS: interventricular septum thickness, PW: posterior wall thickness, LVEF: left ventricular ejection fraction, LVMI: left ventricular mass index.

Table 3. Genotype and allele distributions of ACE I/D, angiotensinogen M235T, A-20C, and G-6A polymorphisms among the groups

	AF (+) patients (n = 150)	Control group 1 AF (-) patients (n = 100)	Control group 2 Healthy controls (n = 100)	p
ACE I/D polymorphism				
D allele (n, %)	185 (61.7%)	107 (53.5%)	116 (58.0%)	0.192
I allele (n, %)	115 (38.3%)	93 (46.5%)	84 (42.0%)	0.192
DD genotype (n, %)	61 (40.7%)	40 (40.0%)	41 (41.0%)	0.989
ID genotype (n, %)	63 (42.0%)	27 (27.0%)	34 (34.0%)	**0.049**
II genotype (n, %)	26 (17.3%)	33 (33.0%)	25 (25.0%)	**0.017**
Angiotensinogen M235T polymorphism				
M allele (n, %)	128 (42.7%)	113 (56.5%)	96 (48.0%)	**0.010**
T allele (n, %)	172 (57.3%)	87 (43.5%)	104 (52.0%)	**0.010**
MM genotype (n, %)	33 (22.0%)	24 (24.0%)	9 (9.0%)	**0.011**
MT genotype (n, %)	62 (41.3%)	65 (65.0%)	78 (78.0%)	**< 0.001**
TT genotype (n, %)	55 (36.7%)	11 (11.0%)	13 (13.0%)	**< 0.001**
Angiotensinogen A-20C polymorphism				
A allele (n, %)	220 (73.3%)	145 (72.5%)	141 (70.5%)	0.784
C allele (n, %)	80 (26.7%)	55 (27.5%)	59 (29.5%)	0.784
AA genotype (n, %)	82 (54.7%)	52 (52.0%)	52 (52.0%)	0.885
AC genotype (n, %)	56 (37.3%)	41 (41.0%)	37 (37.0%)	0.803
CC genotype (n, %)	12 (8.0%)	7 (7.0%)	11 (11.0%)	0.568
Angiotensinogen G-6A polymorphism				
A allele (n, %)	127 (42.3%)	142 (71.0%)	133 (66.5%)	**< 0.001**
G allele (n, %)	173 (57.7%)	58 (29.0%)	67 (33.5%)	**< 0.001**
AA genotype (n, %)	41 (27.3%)	52 (52.0%)	45 (45.0%)	**< 0.001**
GA genotype (n, %)	45 (30.0%)	38 (38.0%)	43 (43.0%)	0.098
GG genotype (n, %)	64 (42.7%)	10 (10.0%)	12 (12.0%)	**< 0.001**

Genotype and allele distributions of ACE I/D, angiotensinogen M235T, A-20C and G-6A polymorphisms among the groups are shown in table 3. The frequency of II genotype of ACE I/D polymorphism was significantly lower in patients with AF compared with the controls. II genotype was associated with a lower risk of AF ($p = 0.012$, odds ratio (OR): 0.514, 95% confidence interval (CI): 0.305–0.865). Patients with AF had a higher frequency of angiotensinogen M235T polymorphism T allele and TT genotype than the control groups. M235T polymorphism T allele increased the risk of AF by 1.470 ($p = 0.012$, 95% CI: 1.088–1.988) while TT genotype increased AF risk by 4.246 ($p < 0.001$, 95% CI: 2.473–7.289). There was no significant association between AF and angiotensinogen A-20C polymorphism. Patients with AF had significantly higher frequency of angiotensinogen G-6A polymorphism G allele and GG genotype compared with the controls. Angiotensinogen G-6A polymorphism G allele and GG genotype increased AF risk by 2.997 ($p < 0.001$, 95% CI: 2.194–4.093) and 6.021 ($p < 0.001$, 95% CI: 3.479–10.421), respectively.

We modelled a logistic regression analysis to explore the independent predictors of AF. Gender, age, hypertension, diabetes, ischemic heart disease, left atrial diameter, LVMI, presence of valvular pathology, ACE I/D polymorphism (I allele and II genotype), angiotensinogen M235T polymorphism (T allele and TT genotype), angiotensinogen A-20C polymorphism and G-6A polymorphism (G allele and GG genotype) were included in the model. The adjusted R square value of the model was 0.724 and the p value was < 0.001. Logistic regression analysis revealed that ACE I/D polymorphism II genotype, angiotensinogen M235T polymorphism TT genotype, and G allele and GG genotype of angiotensinogen G-6A polymorphism were independent predictors of AF in addition to enlarged left atrium, LVMI, and valvular pathology in our cohort (table 4).

Discussion

Atrial fibrillation is the most commonly observed arrhythmia in clinical practice and there is a growing effort to prevent AF, which brings out the importance of understanding the underlying mechanisms for the development of AF. Since the activated local atrial RAS and increased ACE expression in the atrial tissue have been reported to play an important role in the pathogenesis of AF,[12,14] it is suggested that RAS genes might be among the susceptibility genes of non-familial structural AF. Tsai *et al.*[30] reported for the first time the associations between RAS gene polymorphisms and non-familial structural AF. They explored the

Table 4. Multivariate regression analysis for predictors of atrial fibrillation

	P	Odds ratio	95% Confidence interval
Female gender	0.652	1.203	0.539–2.687
Age	0.559	1.010	0.976–1.045
Hypertension	0.583	1.287	0.524–3.159
Diabetes	0.494	0.747	0.323–1.723
Ischemic heart disease	0.091	0.493	0.217–1.119
Left atrial diameter > 40mm	**< 0.001**	16.846	6.694–42.391
Left ventricular mass index	**0.009**	1.009	1.002–1.015
Valvular pathology	**< 0.001**	8.404	3.694–19.119
ACE I/D polymorphism I allele	0.363	1.475	0.638–3.408
ACE I/D polymorphism II genotype	**0.006**	0.261	0.101–0.676
Angiotensinogen M235T polymorphism T allele	0.219	1.790	0.707–4.533
Angiotensinogen M235T polymorphism TT genotype	**< 0.001**	5.825	2.257–15.030
Angiotensinogen A-20C polymorphism AC genotype	0.609	0.817	0.377–1.773
Angiotensinogen G-6A G allele	0.039	2.358	1.043–5.329
Angiotensinogen G-6A polymorphism GG genotype	**< 0.001**	6.794	2.541–18.167

associations of ACE gene I/D polymorphism, the T174M, M235T, G-6A, A-20C, G-152A, and G-217A polymorphisms of the angiotensinogen gene, and the A1166C polymorphism of the angiotensin II type I receptor gene in a total of 250 patients with documented non-familial structural AF and 250 controls. They found that M235T, G-6A, and G-217A were significantly associated with AF in single-locus analysis.

The novel finding of our study was the demonstration of association of AF with the RAS gene polymorphisms in a Turkish population for the first time. We found that the frequency of II genotype of ACE I/D polymorphism was significantly lower in patients with AF, suggesting a protective role of II genotype for AF. This seems plausible since D allele is associated with a higher plasma and cardiac ACE activity.[20,37,38] Furthermore, several studies have reported that D allele increases the risk of sudden cardiac death and arrhythmia including AF and DD/ID genotypes is associated with failure of antiarrhythmic drug therapy.[29,39,40] Although the study from Taiwan reported that there was no relation between AF and ACE I/D polymorphism[30], Ravn *et al.*[31] reported that ACE I/D genotype, in combination with angiotensinogen A-20C genotype, predicted an increased risk of AF in a Danish population. Fatini *et al.*[28] also highlighted the role of ACE gene in predisposing to both lone and secondary non-valvular AF.

In our study, we also found that polymorphisms in the angiotensinogen gene were associated with AF. The frequencies of angiotensinogen M235T polymorphism T allele and TT genotype and G-6A polymorphism G allele and GG genotype were higher in AF patients compared with the controls. Tsai *et al.*[30] found that frequencies of the M235 allele in exon 2 and G-6 allele in the promoter region of the angiotensinogen gene were significantly higher in cases with AF than the controls. They reported that the odds ratios for AF were 2.5 (95% CI 1.7–3.3) with M235/M235 plus M235/T235 genotype and 3.3 (95% CI 1.3–10.0) with G-6/G-6 genotype. However, Ravn *et al.*[31] found no relation between AF and G-6A polymorphisms. Instead, they reported that CC genotype of A-20C polymorphisms increased the risk of AF. However, similar to the study of Tsai *et al.*,[30] we did not find any association between AF and A-20C polymorphism.

We believe that the difference in the results might be based on the difference in the genetic composition of the populations. In addition, Tsai *et al.*[30] reported that they detected significant gene–gene interactions by the multifactor-dimensionality reduction method and multilocus linkage disequilibrium tests while they were exploring the association between polymorphisms in RAS genes and AF. For instance, they suggested that there was no functional significance of the M235T polymorphism and the association of the M235 allele with AF might be through its tight linkage with the G-6 allele.[41,42] Thus, the gene–gene interactions might also explain the differences in the results.

Control subjects in our study consisted of both healthy individuals and patients with diseases, such as diabetes, hypertension, or coronary artery disease. The only difference between the AF group and the control group consisting of patients with other diseases was the presence or absence of AF. Although the groups were similar in respect of age, gender, hypertension, diabetes, and coronary artery disease, AF patients had significantly larger left atrium, higher LVMI, and higher frequency of significant valvular pathology. These findings may imply that stretching of the atria is responsible for the development of AF. In the Framingham study, it was found that having a valvular heart disease or left atrial dilation increased the risk of AF.[43,44] In our study, enlarged left atrium, LVMI, and

presence of significant valvular pathology were also found as independent predictors of AF. Because left atrial size, LVMI, and presence of significant valvular pathology were not balanced in cases and the controls, we reanalysed the associations between AF and RAS polymorphisms with adjustment for these parameters. We found that ACE I/D polymorphism II genotype, angiotensinogen M235T polymorphism TT genotype, and G allele and GG genotype of angiotensinogen G-6A polymorphism were still independent predictors of AF in our cohort.

Study limitations

There are certain limitations to our study. First, the present results provide evidence only of the association between RAS gene variations and AF at the gene level and do not demonstrate the direct mechanism by which RAS causes AF. Second, this study is a cross-sectional study with a small sample size. There is surely a necessity for more ample sampling and we believe that prospective follow-up of patients with sinus rhythm and determination of which patients developed AF finally would yield better data about the possible associations between RAS genes and AF. Third, we could not explore gene–gene interactions. As many authors noted, sorting out the nature of the interactions in multidimensional space to infer function remains an interpretive challenge.[30] Last, we did not exclude patients with lone AF in our study. The cause of lone AF may be primarily an ionic mechanism,[45] and the role played by RAS may not be as important as in secondary structural AF.

Conclusion

Activation of the RAS may be an important risk factor for the development of AF. Patients who have a specific genetic variation or polymorphism in the RAS genes may be more liable to develop AF. We found that ACE I/D polymorphism II genotype, angiotensinogen M235T polymorphism TT genotype, and G allele and GG genotype of angiotensinogen G-6A polymorphism were independent predictors of AF in addition to enlarged left atrium, LVMI, and presence of significant valvular pathology in our cohort. This is the first study conducted in a Turkish population, but its applicability to the general population or to different populations is uncertain and warrants further, if possible prospective, study with a larger sample size.

Funding

This work was supported by the Marmara University Research Fund.

Conflict of interest statement

None declared.

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