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Renin–angiotensin system genetic polymorphisms: Lack of association with CRP levels in patients with coronary artery disease

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Abstract

Angiotensin (Ang) II is believed to be a potential pro-inflammatory factor. The capability of Ang II to stimulate C-reactive protein (CRP) production has recently been described. Genetic polymorphisms of renin angiotensin system (RAS) components have been described to be associated with the development of coronary artery disease (CAD). This study investigated the association between six different genetic polymorphisms of RAS and serum CRP levels in a sample of CAD patients. Genotyping of RAS genes polymorphisms in 176 patients with documented CAD was performed by a modified polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Measurement of high-sensitivity (hs)-CRP was performed using standard immunoturbidimetric methods. Results show no significant differences in serum CRP regarding different variants of the six polymorphisms studied (p = 0.41, 0.24, 0.25, 0.19, 0.29, and 0.05 for Ang-converting enzyme (ACE) insertion/deletion (I/D), A-240T and A2350G, angiotensinogen M235T, AT1 receptor A1166C, and AT2 receptor C3123A polymorphisms, respectively). In conclusion, genetic polymorphisms of RAS are not associated with increased serum CRP in CAD. Compensation of an increased activity of ACE through counter-regulation and the secretion of CRP under the influence of Ang II in the vessel being local could explain the lack of association between the studied polymorphisms and CRP levels in CAD patients.

Keywords

Renin–angiotensin system, angiotensin-converting enzyme, coronary artery disease, genetic polymorphism, single nucleotide polymorphism, C-reactive protein

Introduction

The renin–angiotensin system (RAS) is one of the widely studied systems involved in the pathophysiology of coronary artery disease (CAD).[1] Angiotensin II (Ang II), as the major product of the RAS, is believed to be involved in the development of other cardiovascular (CV) pathologies including hypertension, heart failure, and cardiac and vascular fibrosis.[2–4]

Ang II is also known to be a potential pro-inflammatory factor since it induces the release of cytokines such as interleukin-6[5] and tumor necrosis factor-α.[6] Concordantly, angiotensin-converting enzyme (ACE) inhibitors and Ang II receptor type I (AT1) blockers (ARBs) are known to modulate the inflammatory and thus possibly the atherosclerotic processes.[7] It is inferred that Ang II may have the capability of eliciting inflammation in the process of atherogenesis. Several reports have shown that inflammation plays a major role in the various stages of atherosclerosis. As a result, inflammatory markers are attracting more attention as the possible risk predictors of disease onset and progression. Among the markers of inflammation, C-reactive protein (CRP) is the most widely studied. CRP classically emanates from the liver; however, it may also be produced

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by vascular sources, including cells residing in atherosclerotic plaque.[8] A large body of studies suggest that CRP is involved in many processes of atherogenesis such as endothelial cell dysfunction, smooth muscle cell migration, proliferation, and formation of the fibrous cap.[9] Hence, it is emergently recognized that CRP is not merely a marker, but also plays a direct role in atherosclerosis. The usefulness of hs-CRP measurements in the prediction of incident myocardial infarction (MI),[8] stroke,[10,11] peripheral arterial disease,[12] and sudden cardiac death[13] has been reported. CRP levels of < 1, 1 to 3, and > 3 mg/l correspond to low-, moderate-, and high-risk groups for future CV events.[11] So having been proven to be a strong independent predictor of incident CAD,[14–16] and in light of the data supporting the usefulness of hs-CRP measurements for short-term prognosis and long-term risk assessment after a CV event,[10,11,17–19] it can be assumed that every new marker of risk should be in agreement with hs-CRP measurements.

Interestingly, the crosstalk between Ang II and CRP in the vascular wall has recently been described. Peng et al. in a study provided evidence that Ang II is capable of inducing CRP generation in vascular smooth muscle cells (VSMCs) in vitro and in vivo, which is mediated predominantly through AT1 receptors in the VSMCs and followed by mitogen-activated protein kinase (MAPK) activation.[20] Subsequently, Han et al. also showed that Ang II stimulates human aortic endothelial cells to produce CRP in mRNA and protein levels.[21] On the other hand, CRP itself is also capable of up-regulating AT1 receptors in vascular smooth muscles,[22] thus demonstrating that there is crosstalk between Ang II and CRP.

Production of Ang II is mainly through the catalytic activity of ACE. Genes coding for ACE and other components of RAS, as a result, are the major determinants of Ang II expression and effect. Genetic variations in the components of RAS, though not yet proven to be helpful as a marker of risk, may explain the familial susceptibility to atherosclerotic processes, especially CAD in certain populations. Among the ACE polymorphisms, some previous studies suggested that ACE insertion/deletion (I/D),[23] A-240T,[24] and A2350G[25] exert significant impact on serum ACE concentration. AT1 receptor might also be implicated in CAD by various mechanisms. Likewise, the relationship between A1166C polymorphism of AT1 receptor and CAD has been reported.[26,27] AT2 receptor, too, is believed to increase under some pathological conditions such as hypertension, vascular injury, and stroke.[28] Association of the genetic polymorphism of AT2 receptor (C3123A) and CAD has been previously reported as well.[29,30]

Considering these findings, we hypothesized that changes in ACE activity brought about by the ACE I/D and other polymorphisms of ACE genes studied here (A-240T and A2350G) as well as alterations caused by angiotensinogen (AGT), AT1 and AT2 genetic polymorphisms (M235T, A1166C, and C3123A, respectively) could result in an increase in hs-CRP levels in diseased vessels. This study was conducted to further understand the outcome of these genetic variations in an attempt to relate the genetic polymorphisms in RAS to the level of risk in patients with CAD. More important, in trying to link the genetic polymorphisms in RAS to the inflammatory component of atherosclerosis, we aimed to provide other evidence explaining the previously reported association of polymorphisms in the ACE gene with CAD.[31,32] To the best of our knowledge, this is the first report on the association of six different polymorphisms of the RAS and CRP level in a sample of atherosclerotic CAD patients.

Methods and materials

Subjects were 176 patients with chronic stable angina who were scheduled to undergo coronary arteriography at the cardiologists' discretion (135 patients because of high-risk results of non-invasive tests including Duke treadmill score < –11 or multiple reversible or large perfusion defects on cardiac scintigraphy, and 41 patients because of their high-risk clinical profile). Those with the diagnosis of acute coronary syndrome, history of active cigarette smoking more than one pack/year, as well as patients taking chronic ACE inhibitor medication (> one month) or with signs of renal insufficiency (serum creatinine > 1.5 mg/dl) or active infectious or inflammatory process were excluded because of the possible impact on the study parameters. The patients were evaluated according to a detailed study protocol containing anamnestic and clinical information about CAD risk factors. All subjects gave informed consent.

On coronary arteriography, the diagnosis of obstructive CAD was made as the presence of at least one stenotic lesion in at least one major coronary vessel or one of its major branches, compromising more than 50% of the vessel's diameter by visual assessment.

Fifteen milliliters of blood were obtained from the arterial access during angiography before the contrast was injected. Genomic DNAs were extracted from whole blood leukocytes using a salting out method.[33] The extracted DNAs were solved in sterile distilled water and stored at 4ºC for further polymerase chain reaction (PCR) analysis. PCR amplification/detection of ACE I/D was carried out using standard protocol.[34] In order to avoid mistyping ID as DD genotype, all DD genotypes were reconfirmed by another typing system.[35] PCR amplification of A-240T, A2350G, M235T, A1166C, and C3123A was performed using primers mentioned in Table 1.[25,34,36–40] In each reaction, 100–200 ng of genomic DNA was amplified in 15 µl of 1×PCR master mix (67 mM Tris base, pH 8.8, 16.6 mM (NH4) 2SO4, 2 mM MgCl2, 0.1% Tween-20, 200 µM deoxyribonucleotide triphosphates (dNTPs), 5% glycerol, 100 µg/ml cresol red) containing 0.2–2.0 µM of each primer and 0.5 U of Taq DNA polymerase (Cinnagen Inc, Tehran, Iran). All genes were amplified under the same procedure,

Table 1. Primers, PCR conditions, and locations of ACE I/D, A-240T, A2350G, M235T, A1166C, and C3123A polymorphisms on DNA.

Polymorphisms	Primer sequence (5'-3')	Location	Restriction enzyme digestion	Allele	DNA fragment size (bp)	References
ACE I/D	F-CTG GAG ACC ACT CCC ATC CTT TCT	Intron 16	None	I	490	(Rigat et al., 1992)[34]
	R- GAT GTG GCC ATC ACA TTC GTC AGA T			D	290	
	F-TTT GAG ACG GAG TCT CGC TC			I	408	(Shanmugam et al., 1993)[35]
	R- GAT GTG GCC ATC ACA TTC GTC AGA T					
A-240T	F- TCG GGC TGG GAA GAT CGA GC	5'UTR	*Xba*I at 37 ^{0C}/24 h	A	137	(Hsieh et al., 2005)[36]
	R- GAG AAA GGG CCT CCT CTC TCT			T	114+23	
A2350G	F-CTG ACG AAT GTG ATG GCC GC	Intron 17	*Bst*UI at 60 ^{0C}/24 h	A	122	(Iqbal et al., 2004)[37]
	R-TTG ATG AGT TCC ACG TAT TTC G			G	100+22	
M235T[a]	F-CAG GGT GCT GTC CAC ACT GGA CCC C	Exon 2 (+704)	*Pfl*FI at 37 ^{0C}/ 24 h	M	165	(Russ et al., 1993)[38]
	R-CCG TTT GTG CAG GGC CTG GCT CTC T			T	140+25	
A1166C	F-ATA ATG TAA GCT CAT CCA CC	3' UTR	*Dde*I at 37 ^{0C}/24 h	A	367	(Takami et al., 1998)[39]
	R-GAG ATT GCA TTT CTG TCA GT			C	224+143	
C3123A	F-GGA TTC AGA TTT CTC TTT GAA	Chromosome X	*Alu*I at 37 ^{0C}/24 h	C	321	(Katsuya et al., 1997)[40]
	R-GCA TAG GAG TAT GAT TTA ATC			A	214+107	

[a]Diamino acid polymorphism; PCR: polymerase chain reaction; ACE I/D: angiotensin-converting enzyme insertion/deletion; M: methionine; T: threonine.

which is of great advantage to reduce workload. The program under which the amplification took place was a modified form of the previous studies. After initial denaturation at 96ºC for 2 min, PCR was carried out for five cycles, each one composed of denaturation at 96ºC for 40 seconds (s), annealing at 60ºC for 50 s and extension at 72ºC for 30 s followed by 25 cycles of denaturation at 96ºC for 40 s, annealing at 55ºC for 50 s and the extension at 72ºC for 30 s. An Eppendorf gradient Mastercycler (Hamburg, Germany) PCR machine was used as the thermal cycler. PCR products (7 µl) were digested with the specified enzymes mentioned in Table 1. Digested fragments were separated by electrophoresis on 3% agarose (Invitrogen® UltraPure) gel after an overnight incubation (Table 1). They were then stained by ethidium bromide and visualized in an ultraviolet (UV) transilluminator. All DNA samples were genotyped at least twice and the results were reconfirmed.

The measurement of hs-CRP was performed using standard immunoturbidimetric methods with a measuring range of 0.20–1440 mg/l.

Statistical analysis

The data were analyzed using SPSS® 16.0 for Windows® (SPSS Inc, Chicago, Illinois). All continuous variables are presented as means ± standard deviation (S.D.). Allele and genotype frequencies are expressed in percentage (%). Distribution of all continuous variables was tested for normality with the Kolmogorov-Smirnov test. Hardy-Weinberg equilibrium (HWE) for the distributions of genotypes was estimated by the chi square (χ^2) test. The relationship between each ACE, angiotensinogen, AT1 and AT2 genes polymorphisms and serum CRP level was analyzed using one-way analysis of variance (ANOVA). Pair-wise

Table 2. Mean and standard deviation of common atherosclerosis risk factors in the study population.

	Mean ± S.D.[a]	
Age	61.93 ± 9.36	years old
Total cholesterol	176.8 ± 39.6	mg/dl[b]
LDL cholesterol	89.3 ± 27.0	mg/dl
HDL cholesterol	40.9 ± 9.2	mg/dl
TG	181.3 ± 91.3	mg/dl
FBS	122.2 ± 45.3	mg/dl
Cr	1.24 ± 0.34	mg/dl
Uric acid	5.63 ± 3.44	mg/dl
Systolic BP	129.0 ± 19.4	mmHg[c]
Diastolic BP	79.5 ± 8.7	mmHg
Body mass index	28.14 ± 4.27	kg/m^2[d]

[a]Standard deviation; [b]milligram per deciliter, [c]millimeters of mercury, [d]kilograms per square meter. LDL: low-density lipoprotein; HDL: high-density lipoprotein; TG: triglycerides; FBS: fasting blood sugar; Cr: serum creatinine; BP: blood pressure.

comparisons were performed by Bonferroni's post-hoc test. Investigation of the effect of polymorphisms on serum hs-CRP was performed using multiple linear regression models by means of Stata® software, version 8SE (Stata Inc, TX, USA). For all the analyses, statistical significance was considered as $p < 0.05$.

Results

Seventy-three women (41%) and 103 men (59%) were included in the study, with mean age of 61.9 ± 9.3 years (from 37 to 86 years old). Table 2 shows the distribution of common atherosclerosis risk factors in the study population. Eighty-eight patients gave a history of hypertension while the remainder were normotensives. Genotype distribution of the study population is listed in Table 3.

The measurements of hs-CRP included values from 0.15 to 32.4 mg/l (mean = 5.58 ± 4.72 mg/l).

There were no significant differences in hs-CRP measurements between wild type, heterozygote, and mutant forms of any of the six polymorphisms studied ($p = 0.41$, 0.24, 0.25, 0.19, 0.29, and 0.05 for ACE I/D, A-240T, A2350G, M235T, A1166C, and C3123A polymorphisms, respectively). Categorizing patients as low, intermediate, and high risk based on their hs-CRP measurements of < 1 mg/l, 1–3 mg/l and ≥ 3 mg/l, respectively, we repeated the statistical test to find an association with any of the polymorphisms; however, there were no significant differences in the three genotypes of any of the six polymorphisms ($p = 0.53$, 0.90, 0.61, 0.77, 0.39, and 0.16 for ACE I/D, A-240T, A2350G, M235T, A1166C, and C3123A polymorphisms, respectively). Considering the equal number of patients with hs-CRP values of < 3 mg/l and ≥ 3 mg/l in this study (88 patients), no significant difference was again observed in the distribution of the high-risk and cumulative low- and intermediate-risk groups with respect to wild, heterozygote, and mutant forms of the genotypes ($p = 0.48$ for ACE I/D polymorphism). Table 4 summarizes the results of the association analysis between hs-CRP (mean and groups) and genetic polymorphisms.

Adjusting for common CV risk factors and using multiple regression analysis, we also found no association between each polymorphism and CRP level in these patients.

Table 3. Genotype distribution of the study population.

Genotype		Frequency (%)	Genotype		Frequency (%)
ACE I/D	W	22.0	A2350G	W	24.7
	M	35.8		M	24.1
	H	42.2		H	51.1
M235T	W	22.7	A1166C	W	68.4
	M	31.4		M	2.3
	H	45.9		H	29.3
A-240T	W	33.9	C3123A	W	56.9
	M	13.2		M	3.4
	H	52.9		H	39.7

ACE I/D: angiotensin-converting enzyme insertion/deletion; W: wild type; H: heterozygote; M: mutant.

Discussion

RAS is definitely involved in the atherosclerotic process. Ang II especially has a strong atherogenic effect.[41,42] Moreover, there are recent reports that Ang II stimulates VSMCs and endothelial cells to produce CRP.[20,21] This locally secreted CRP is believed to play a direct and essential role in the whole inflammatory process of atherosclerosis and the development of CV complications.[43] AT1 receptors, reported to be present on the endothelial cells,[44] may regulate the proinflammatory function of Ang II. CRP itself is also capable of up-regulating the AT1 receptors in VSMCs.[22]

As the major source of Ang II production in serum and tissues, enzymatic activity of ACE and its plasma concentrations are genetically determined by ACE gene polymorphisms.[23,34,45] There are reports that the ACE I/D genetic polymorphism accounts for 47% of phenotypic variance in ACE plasma levels. Homozygotes for the D allele show an 85% increase in ACE activity compared to I allele homozygotes.[23]

An association of ACE I/D gene polymorphism as well as polymorphisms in the other components of RAS with increased risk of CAD in various populations has been controversial. Several investigators found no association with the occurrence of either CAD, MI[46–50] or long-term clinical outcomes,[51] while others demonstrated a positive association between especially the D allele and ischemic heart disease generally or for certain subgroups of patients.[31,37,46,48–50,52–54] Assessing six different genetic polymorphisms of the RAS components in a group of CAD patients was based on the study hypothesis that the influence of genetic polymorphisms on the activity of ACE or

Table 4. Results of the association analysis between hs-CRP (mean and groups) and genetic polymorphisms.

Genetic polymorphism		hs-CRP (mg/lit)				*p* value
		Mean (CI 95%)	< 1 (%)	1–3 (%)	≥ 3 (%)	
ACE I/D	W	5.9 (3.6–8.2)	18.4	28.9	52.6	0.532
	M	4.1 (2.9–5.3)	12.9	46.8	40.3	
	H	4.7 (3.4–6.1)	15.1	41.1	43.8	
A-240T	W	6.3 (4.4–8.3)	15.3	35.6	49.2	0.899
	M	3.8 (2.1–5.5)	17.4	43.5	39.1	
	H	4 (3.1–4.9)	14.1	42.4	43.5	
A2350G	W	5.7 (3.7–7.8)	14.0	34.9	51.2	0.608
	M	3.8 (2.4–5.2)	11.9	50.0	38.1	
	H	4.7 (3.6–5.9)	16.9	38.2	44.9	
M235T	W	3.7 (2–5.4)	20.5	38.5	41.0	0.766
	M	5.3 (3.5–7)	14.8	40.7	44.4	
	H	5 (3.8–6.2)	11.4	40.5	48.1	
A1166C	W	4.7 (3.7–5.7)	12.6	42.9	44.5	0.390
	M	5.8 (2–9.7)	0.0	25.0	75.0	
	H	4.8 (3–6.6)	21.6	35.3	43.1	
C3123A	W	4.7 (3.6–5.7)	13.1	39.4	47.5	0.162
	M	1.3 (0.2–2.4)	50.0	33.3	16.7	
	H	5.2 (3.7–6.7)	14.5	42.0	43.5	

hs-CRP: high-sensitivity C-reactive protein; ACE I/D: angiotensin-converting enzyme insertion/deletion; W: wild type; H: heterozygote; M: mutant; CI: confidence interval.

Ang II receptors could show measurable effects on CRP plasma levels in atherosclerosis. This could even provide clues relating ACE I/D genetic polymorphism to the inflammatory component of atherosclerosis in patients with CAD. Furthermore, it is known that CRP itself not only has an independent predictive value for future CV events in chronic CAD, it also can add prognostic information at all levels of Framingham risk score.[8,11,15] Thus, finding an association between the genetic variations in RAS components and CRP plasma levels could theoretically relate each genetic polymorphism to the level of risk in CAD patients.

In the present study, serum concentration of CRP was not associated with ACE I/D polymorphism in CAD patients. Neither was it associated with ACE A-240T and A2350G, AGT M235T, AT1 receptor A1166C and AT2 receptor C3123A genetic polymorphisms. Other authors were also unable to relate plasma levels of renin, Ang II, aldosterone, endothelin-1, and vasopressin with ACE I/D genetic polymorphism.[55–58] The association of ACE I/D polymorphism with CRP as a probable RAS-related peptide and also a marker of inflammation and worse prognosis in CAD has not been investigated previously. However, this study found no association of the ACE I/D polymorphism with plasma levels of CRP in patients with CAD. An increased activity of ACE, genetically determined by the D allele of ACE I/D polymorphism in certain populations, may be compensated within the RAS or via non-ACE pathways of Ang II generation through counter-regulation.[59] This could explain why CRP plasma levels show no difference between genotype subgroups. On the other hand, a report in a healthy Iranian population demonstrated that ACE activity was only minimally increased in carriers of D allele.[60] Another explanation is that the secretion of CRP under the influence of Ang II in the vessel wall[20,21] most probably would have a local paracrine effect and not necessarily lead to an elevated concentration of CRP in plasma.

Although there are several reports on the association between the ACE I/D genetic polymorphism with CAD in different populations,[31,46,48,49,52–54] available meta-analyses have shown only a modest association.[61,62] Moreover, while prospective data regarding the CAD clinical outcome and future CV events in the carriers of the reported high-risk genotype, which is the D allele homozygotes, are lacking, plasma hs-CRP levels of more than 3 mg/l can categorize such gene carriers as at high risk of having a future CV event.[8,11] Elevated CRP in patients with CAD is even believed to be a stronger predictor than low-density lipoprotein (LDL) cholesterol.[15] High-risk patients are believed to have more vulnerable plaques and are more likely to develop fatal and non-fatal MIs.[11] On the other hand, the effect of Ang II on atherosclerotic plaque development and progression does not necessarily translate into the plaque being vulnerable to rupture or erosion. Thus, elevated CRP plasma level shows the plaque vulnerability and not the extent of the atherosclerosis, and Ang II acts more on the atherosclerotic plaque development, not necessarily making it vulnerable. Association of ACE I/D polymorphism with CAD, but not with CRP plasma level, can be viewed in this regard as well, as it shows an increased probability for CAD development and not CV events, the theory that needs verification in a prospective study in the future.

Conflict of interest

The authors report no conflicts of interest.

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