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Association study of the angiotensin-converting enzyme (ACE) gene G2350A dimorphism with myocardial infarction

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Abbreviations: ACE, angiotensin-converting enzyme; AMI, acute myocardial infarction; BMI, body mass index; CVD, cardiovascular disease; df, degrees of freedom; I/D, insertion/deletion; IHD, ischemic heart disease; LD, linkage disequilibrium; QTL, quantitative trait locus

Abstract

The angiotensin converting enzyme (ACE) is a strong candidate gene for myocardial infarction (MI). Insertion-deletion dimorphism in intron 16 of this gene has been inconclusively found to be associated with it. Several new polymorphisms in the *ACE* gene have been identified and among these, a dimorphism in exon 17, *ACE* G2350A, has a significant effect on plasma ACE concentrations. To assess the value of genotyping the *ACE* G2350A dimorphism in a genetically homogeneous population, we carried out a case-control study of dimorphism G2350A for a putative association with MI among Pakistani nationals. We investigated a sample population of 370 Pakistanis, comprising 163 controls, and 207 patients with clinical diagnosis of acute MI (AMI). *ACE* G2350A alleles were visualized by assays based on polymerase chain reaction and restriction endonuclease analysis. Frequencies of G alleles were 0.68 among controls and 0.72 among AMI patients. The *ACE* G2350A dimorphism showed no significant association with MI (χ^2 = 0.90, 2 df, *P* = 0.64), plasma levels of homocysteine (*P* = 0.52) or with serum levels of folate (*P* = 0.299). The results indicate that *ACE* G2350A polymorphism is not associated with risk of myocardial infarction in the Pakistani population investigated here.

Keywords: angiotensin converting enzyme; folic acid; genetics; homocysteine; myocardial infarction; Pakistani population

Introduction

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxypeptidase I, (EC. 3.4.15.1) that activates angiotensin I through cleavage of the carboxyterminal dipeptide into the potent vasoconstrictor angiotensin II and inactivates the vasodilator peptide bradykinin (Erdös and Skidgel, 1987). Both are mediators of vascular tone and smooth muscle cell proliferation (Ehlers and Riordan, 1989). Experimental data suggest that the presence of high levels of plasma ACE could result in the thickening of the vascular wall eventually leading to the development of vascular disease (Erdös and Skidgel, 1987; Ehlers and Riordan, 1989).

Circulating ACE levels show extensive inter- individual variability and are highly genetically determined (Cambian *et al.,* 1988; Alhenc-Gelas *et al.,* 1991). An insertion/deletion (I/D) dimorphism, due to the presence or absence of a 287 base pair (bp) *alu*-type sequence in intron 16 of the *ACE* gene, has been shown to cosegregate with serum and tissue ACE activities, and major locus inheritance explains best the findings that the D allele is associated with elevated ACE levels (Cambian *et al.,* 1988; Rigat *et al.,* 1990; Alhenc-Gelas *et al.,* 1991). Thus, the *ACE* gene is viewed as a quantitative trait locus (QTL) that modulates circulating ACE levels, and the *ACE* I/D dimorphism is a marker that is thought to be in linkage disequilibrium (LD) with functional variants located in the *ACE* gene (Cambian *et al.,* 1992; Tiret *et al.,* 1992; Arca *et al.,* 1998) that are implicated in CVDs.

Several studies on the association of *ACE* I/D polymorphism and the risk for myocardial infarction (MI) (Bohn *et al.,* 1993; Cambien *et al.,* 1994; Evans *et al.,* 1994; Soubrier and Cambien, 1994; Cambian and Evans, 1995; Lindpainter *et al.,* 1995; Villard and Soubrier, 1996; Ferrieres *et al.,* 1999; O' Mally *et al.,* 1999; Keavney *et al.,* 2000), have generated incon-

sistent information. This has stimulated a search for new polymorphisms in the *ACE* gene to identify better markers or actual functional variants. Accumulated evidence points to the existence of two QTLs at this chromosomal locus (McKenzie *et al.,* 1995; Villard *et al.,* 1996; Zhu *et al.,* 2000; McKenzie *et al.,* 2001). A genome-scan analysis by The Framingham Heart Study found strong evidence for a QTL on chromosome 17, located close to the *ACE* gene and linked to blood pressure (Levy *et al.,* 2000). Amongst the 13 polymorphisms of the *ACE* gene recently reported, a dimorphism in exon 17, *ACE* G2350A, has the most significant effect on plasma ACE concentrations (Zhu *et al.,* 2001). After adjustment for the effect of *ACE* G2350A dimorphism, the I/D dimorphism was no longer associated with ACE, indicating that it is in LD with *ACE* G2350A and unlikely to be a functional mutation (Zhu *et al.,* 2001).

To assess the value of genotyping of *ACE* in Pakistani population, we carried out a case-control study of dimorphism G2350A for a putative association with MI and with plasma/serum levels of homocysteine and folate amongst Pakistani nationals. Our study is aimed at establishing whether the *ACE* G2350A dimorphism is a genetic marker and an independent risk factor for MI.

Materials and Methods

Study subjects

Two hundred and seven consecutive Pakistani patients with acute MI (AMI) (age, 30-70 years) admitted to the National Institute of Cardiovascular Diseases (NICVD), Karachi from January 2001 to June 2001, were selected for this study. They had confirmed diagnosis of AMI on the basis of clinical history, ECG and biochemical data.

Similarly, 163 normal healthy subjects who had been matched for sex and to some extent for age and belonged to the same socio-economic class, were selected from the personnel of the Aga Khan University and Civil Hospital, Karachi for this study. Informed consent was obtained from the participants and the study was approved by the Ethical Committee of the Aga Khan University.

A stringent criteria was used for the selection of normal healthy control subjects. In addition to being matched for age, sex and socioeconomic background, they had no evidence of CAD, diabetes mellitus, hypertension, obesity and hypercholesterolemia. Those control subjects who were found to be pregnant, using antiepileptics, oral contraceptives, having malabosorption syndrome, suffering from tuberculosis, uremia, liver disease, or using B-complex vitamins during the last six months were excluded from this study.

DNA analysis

Blood was collected in 10 m; Na-EDTA tubes and kept frozen at -20°C. DNA was extracted using standard protocols (Sambrook *et al.,* 1989) and stored in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Codon 2350 *ACE* genotypes were first visualized by a method based on allele-specific oligonucleotides (McKenzie *et al.,* 1995). It was then described as *ACE-8* by Zhu *et al.* (2001), as a modification by introducing a mismatch guanine at the 3' end of the primer sequence, resulting in the amplification of a *BstU*1 restriction site. This more convenient method involves a set of primers designed to amplify a 122-bp fragment encompassing the polymorphic region of *ACE* gene: 5'-CTGACGAATGTGATGGCCGC 3' (upstream) and 5'-TTGATGAGTTCCACGTATTTCG-3' (downstream). The PCR contained 100 to 200 ng DNA template, 125 µM dNTPs, 2.5 mM $MgCl_2$, and 0.3 mM of each primer and 1 U *Taq* polymerase, in a final volume of 10 µl. After initial denaturation at 95°C for 5 min, PCR was carried out for 35 cycles, each one comprised of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension time of 10 min at 72°C. PCR products (5 µl) were digested with 5 U of *BstU*I (Life Technologies) at 60°C for 2 h. Digested fragments were separated by electrophoresis on 3% agarose gel and identified by ethidium bromide staining. Allele G2350 was visualized as a 122-bp fragment and allele A2350 as 100-bp and 22-bp fragments.

Data analysis

Statistical analyses were done with the help of an SPSS® version 10.0 for Windows® software package (Gorinchem, The Netherlands). Distribution differences of G/A 2350 genotypes in the patients (AMI) as compared to distribution in the control group as well as Hardy-Weinberg proportions of allele distribution were assessed by chi-square analyses on 3×2 tables. Means were compared using ANOVA. For all analyses, statistical significance was considered when significance level (*P*) values were lower than 0.05.

Results

Demographic and clinical characteristics of patients have been listed in Table 1. The patient group comprised 51 females and 156 males, showing a female to male ration of 1:3. The mean age was 53± 9.6 years (range 30-70 years) in this group. Mean body mass index (BMI) was 24±5.6, while mean serum glucose concentration was 144±72 mg/dl. Mean cholesterol concentration was 185±45 mg/dl. 43% of them were smokers, 31% were hypertensive,

Table 1. Demographic and Clinical Characteristics of Subjects. (Mean±S.D)

	Controls (*n* = 163)		Patients (*n* = 207)	
	Values	Frequency (%)	Values	Frequency (%)
Age (Years)	49.3±9.6		53±9.6	
Gender (Male)		123 (75.5)		156 (75.4)
(Female)		40 (24.5)		51 (24.6)
Body mass index (kg/m^2)	24.0±5.6		24.5±3.6	
Glucose (mg/dl)	100±24		144±72*	
Cholesterol (mg/dl)	168±34		185±45*	
Smoking status (smokers)		17		43*
(non-smokers)		83		57
Diet (vegetarian)		3		1
(non-vegetarian)		97		99
Parental History of IHD (Yes)		15		26*
(No)			85	74
Hypertension (Yes)		0		31
(No)		100		69
Diabetes (Yes)		0		56
(No)		100		44
Homocysteine	17.8±7.7		17.8±8.2	
Folate	5.33±3.44		3.33±3.81*	

*Indicates a significant difference from the control values ($P < 0.05$).

Table 2. Distribution of *ACE* G2350A genotypes and allele frequencies (±standard errors) in the AMI patients and normal healthy subjects. Percentage values of genotypes are indicated in parentheses.

Genotype	AMI (*n* = 207)	Normal healthy subjects (*n* = 163)
GG	110 (53)	85 (52)
GA	76 (37)	52 (32)
AA	21 (10)	26 (16)
p (G allele)	0.72±0.04	0.68±0.04
q (A allele)	0.28±0.04	0.32±0.04

Table 3. Means (±S.D.) of age, BMI, plasma homocysteine and serum folate values according to ACE G2350A dimorphism in the overall sample population of this study.

Variable	GG (*n* = 195)	GA (*n* = 128)	AA (*n* = 47)	**P*-value
Age (years)	50.1±9.4	50.7±8.9	51.5±8.9	0.609
BMI	24.3±4.4	24.5±4.3	23.6±3.9	0.471
Homocysteine (μmol/l)	18.1±8.4	17.8±8.2	16.6±5.4	0.52
Folate (ng/ml)	3.93±3.6	3.84±3.1	4.71±3.3	0.2991

**P*-value compares the mean values of the three genotypes for each variable by oneway ANOVA.

57% were diabetic, while 19% were hypercholesterolemic. 26% of the patients had parental history of ischemic heart disease (IHD).

Among the controls, the mean age was 49.3±9.6 years (range 31-70 years). There were 123 males and 40 females and the mean BMI for this group was 24 ±5.6 which was quite close to the mean BMI of cases. 17% of the controls were smokers. 15% of the control subjects had parental history of IHD.

Table 2 shows the data pertaining to both genotype and allele distributions in the two groups of subjects. *ACE* genotypes did not occur in Hardy-Weinberg proportions in the normal control group (χ^2 = 5.82, 2 degrees of freedom (df), P = 0.054) nor in AMI patients (χ^2 = 0.90, 2 df, P = 0.64).

Differences in the distributions of the three genotypes according to clinical phenotype were not statistically significant (AMI *vs* Controls: χ^2 = 3.1, 2 df, P = 0.217).

We also sought to explore association of 2350 G > A genotypes (GG, GA, AA) with several phenotypic

variables including age, BMI, serum folate and plasma homocysteine. There was no significant difference between the age and BMI profiles of the three genotypes (Table 3). The homocysteine and serum folate values were also not statistically different between genotypes (Table 3).

Discussion

Association (retrospective case-control) studies are influenced by the effects of selection bias, population stratification, confounding by other variables, and clinical criteria used to define patient groups. Minimizing the influence of the first two variables can be achieved by the exploration of putative associations in various ethnic groups that may be more genetically homogenous.

Pakistani people belong to an ethnic group which is relatively homogeneous and has the high rates of coronary artery disease CAD (McKeigue *et al.,* 1988). Moreover, the relative risk of CAD in South Asian men is the highest at early ages (Balerajan, 1991; McKeigue, 1992). This points to an increased genetic predisposition of this population to the development of CAD.

"Control" individuals that were included in this investigation constituted a "comparison" rather than a "control" group. They were indeed free of disease, sex-matched with the patients, and had similar socioeconomic background to that of the patients. As these control individuals had no evidence of MI, they represented a valid comparison group for these association studies, though it could not be predicted whether some of them would develop MI in future.

Several studies have suggested that the D allele of *ACE* insertion/deletion (I/D) dimorphism confers increased risk for CVD (Cambien *et al.,* 1992; 1994; Villard and Soubrier, 1996). At the same time, considerable negative evidence exists on this question. An analysis of 11,000 cases and controls showed no relationship between MI and the I/D polymorphism (Evan *et al.,* 1994). The I/D marker was likewise not associated with MI in Italian and French sample populations (Arca *et al.,* 1998; Ferrieres *et al.,* 1999). O'Malley *et al.* (1999) summarized the association between the I/D polymorphism and CVD risk, grouping studies by geographical region and disease prevalence. In this analysis the ACE I/D polymorphism did not appear to be a clinically useful indicator of risk for MI. Zhu *et al.* (2001) reported 13 polymorphisms in the *ACE* gene using linkage and association studies. The polymorphism in exon 17, *ACE* G2350A had the most significant effect on plasma ACE concentration, accounting for 19% of the total variance in ACE plasma levels. After adjustment for the effect of *ACE* G2350A dimorphism, the I/D polymorphism was no longer associated with plasma ACE concentration, indicating that it is in LD with *ACE* G2350A and unlikely to be a functional mutation. Besides the effect on plasma ACE concentration, Zhu *et al.* (2001) reported that this dimorphism was significantly associated with SBP with an average increase of 3.2 mmHg with each copy of the G allele. These observations were suggestive of a possible role of *ACE* G2350A polymorphism in MI.

We report the allele frequency of the *ACE* G2350A polymorphism in the Pakistani population to be 0.68± 0.04 and 0.32±0.04 for the G2350 and A2350 alleles respectively. In the present study, G2350A genotype distributions were not in Hardy-Weinberg proportions in the control group nor in the MI patient group. This may be a direct effect of consanguinity in this population.

Our data on the Pakistani population did not show any significant association between G2350A polymorphism and MI. A recent study from our laboratory, reported a significant association between G2350A polymorphism and essential hypertension in Emirati population (Mahmood *et al.,* 2003), indicating that this polymorphism may be the functional mutation of the *ACE* gene. However, no association of G2350A polymorphism was found with MI in Emirati population. Hyperhomocysteinemia has been an established risk factor MI. A recent report by Westphal *et al.* indicated that ACE inhibitors increase the plasma levels of homocysteine in hypertensive patients, thereby compromising to some extent the cardio-protective function usually associated with them (2003). This was also suggestive of a possible interaction between *ACE* gene expression and plasma level of homocysteine. Whether a polymorphic change in the *ACE* gene could have any influence on plasma homocysteine level is unknown, therefore, we investigated whether there is any association between plasma levels of homocysteine and *ACE* G2350A polymorphism. Although we found decreased levels of plasma homocysteine in AA genotype (Table 3), the value was not significantly different from the values in GG and GA genotypes. This is suggestive of no association between any of the three genotypes and plasma levels of homocysteine. Similarly, no association was observed between any of these three genotypes and serum folate levels.

In summary, our data support that the G2350A dimorphism is neither associated with MI nor with plasma homocysteine or folate in the Pakistani population. Given the complex nature of genetic susceptibility for chronic degenerative diseases, further studies need to be conducted in individual ethnic groups to verify the disease relevance of this polymorphism.

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