Full Length Research paper

Implications of I/ D (rs4340) polymorphism in CAD among South Indian population

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Genetic factors are important in the pathogenesis of coronary artery disease (CAD). The I/D polymorphism in the Angiotensin converting enzyme (ACE) gene is a genetic risk factor for CAD patients who have a history of Myocardial Infraction (MI). We investigated the association between I/D polymorphism of the ACE gene and the presence of CAD in one hundred patients (79 males and 21 females, aged between 21- 82) who underwent diagnostic coronary angiography and compared with one hundred patients-as controls (62 males and 38 females, aged between 20- 72) who had false symptoms of CAD. The presence of risk factors including age, hypertension, hypercholesterolemia, tobacco use, diabetes mellitus and hyperuricemia was also determined. ACE I/D polymorphism was detected by polymerase chain reaction. The D allele frequency was higher (p <0-01) in CAD patients. The logistic regression analysis indicated that the D allele in association with classical risk factors had the potential to induce CAD, with odds ratio = 0.58(95% CI; 0.37- 0.90). This study revealed that, the I/D polymorphism of ACE gene (carrying D allele) was found to be an independent risk factor for CAD in the studied South Indian population. The number of risk factors did not alter the frequency of ACE gene genotype among patients with CAD, however, in normotensives, the odds ratio of DD-genotype was significantly higher, as the D allele of ACE gene polymorphism was found to be associated with morbidity in CAD in this study population.

Key words: ACE gene polymorphism, coronary artery disease, myocardial infarction, risk factors.

INTRODUCTION

Cardiovascular diseases represent today the main cause of death in human adults. A reasonable number of studies have focused in testing specific genetic markers among groups of ischemic coronary artery disease (CAD) patients, aiming to find a correlation between these gene polymorphisms and disease. A number of conditions characterized by blood vessel occlusion and/or vascular spasm have been found to be associated in both clinical and epidemiological studies. These include hypertension (HPT), Reynaud's phenomenon (RP), and coronary artery disease (CAD) (Zahavi et al., 1984; Keefe et al., 1993; Brand et al., 1997; Smyth et al., 1999; Nakamura et al., 2000). Whether these associations are the conesquences of a shared environmental risk factor or represent an underlying propensity to develop the conditions

through a common biological mechanism remains controversial. The exploration of the genetic and environmental relationships underlying these conditions is one approach to resolving the biological basis for the association. Atherosclerosis is a systemic dysfunctional endothelial, chronic inflammatory, fibro proliferative, angiogenic, prothrombotic, multifactorial disease of the arterial intima caused by the retention of modified low density lipoproteins, hemodynamic stress, and accelerated by redox stress (Hayden, 2001; Hayden and Tyagi, 2002; Hayden and Tyagi,1998a,b; Hayden and Tyagi, 2000). ACE polymorphism appears to have a significant impact on the progression of CAD. Several studies have found the D allele to be an independent risk factor for CAD. It is important to look for the gene association in the Asian Indian population, in view of the high prevalence of CAD and to see whether the association differs from other populations.

The clinical relevance of ACE gene polymorphism still

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remains unclear, as CAD is a multifactorial disease and the ACE gene alone may not have a direct effect on the severity of CAD and premature death. However, the homozygous DD genotype in older patients (above 50 years of age) was reported to be significant. The present study is important as there is a need for a robust confirmation of the risk gene for CAD, even if the effect is small, so as to contribute to our understanding of the pathology of CAD, and determine potential therapeutic strategies. Angiotensin-1 converting enzyme (ACE) gene is one of the most intensely studied genes because of the key role it plays in the renin–angiotensin system (RAS). ACE catalyses the conversion of angiotensin 1 to angiotensin 2, a vasoactive and aldosterone-stimulating peptide, and inactivates bradykinin (Erdös and Skidgel, 1987) . This gene coding for the angiotensin converting enzyme (ACE) is expressed in several types of somatic cells, including vascular cells, heart, lung, and muscles. It is located on chromosome 17q23 and consists of 26 exons and 25 introns. Intron 16 contains a polymorphism characterized by the presence (insertion (I)) or absence (deletion (D)) of a 287 bp *Alu* repeat sequence, resulting in three genotypes, *DD* and *II* homozygotes and *ID* heterozygote's which has been associated with endurance related phenotypes and the response to training. The I/D polymorphism is reported to determine the circulating and tissue enzyme levels, such that individuals homozygous for the *D* allele have higher tissue and plasma ACE concentrations than heterozygote and *II* homozygote (Rigat et al., 1990; Costerousse et al., 1993). The I/D polymorphism association with cardiovascular diseases has been documented in CAD (Cambien et al., 1992; Marian et al., 1993; Schunkert et al., 1994; Kario et al., 1996), as well as chronic renal diseases (Hohenfellner et al., 2001; Ohtomo et al., 2001).

RAS has been shown to play a key role in the regulation of blood pressure and influence the cardio-vascular system (Van and Pinto, 2003), and several genes belonging to this system have been associated with CAD. Two of the most intensively investigated genetic polymorphisms are the insertion/deletion (I/D) alleles of the angiotensin I-converting enzyme (ACE) gene and mutations at the angiotensin II AT1 receptor. Angiotensinogen (AGT), the precursor of angiotensin II, has been investigated as a candidate gene in the development of the disorder in many studies. Many investigators have reported a positive association of AGT polymorphisms with high blood pressure (Jeunemaitre et al., 1992; Martinez et al., 2002; Pan et al., 2000; Pereira et al., 2003; Sato et al., 2000; Rankinen et al., 2000; Jeunemaitre et al., 1997; Iso et al., 2000).

The main focus of this study was to analyze the ACE gene polymorphism, alone or combined in haplotypes, and clarify their potential association with CAD related conditions. Knowing that in most cases CAD has a multifactorial basis, involving a number of genes and environmental factors interacting to determine whether or not the disease will develop, we also tried to determine a possible interaction between several well known CAD linked risk factors.

MATERIALS AND METHODS

Subjects and methods

Selection criteria: We included 100 patients with angiographically diagnosed CAD pa-tients (79 males and 21 females) consecutively admitted to the hos-pital with proven coronary artery disease (more than 50% stenosis affecting at least one vessel). The controls were those who came to the hospital with pain in the chest but did not have a history of angina pectoris or MI, and they showed a normal electrocardiogram (62 males and 38 females), in whom angiographic examination excluded the presence of coronary artery disease. This study there-fore represents true (cases) and false cases (as controls) of the CAD disease. The study went on for a period of two years during 2006-2008. All the subjects were from southern part of India (Andhra Pradesh andTamil Nadu).

Patient's characteristics: All the patients and controls were interviewed and epidemiological data/demographic data were recorded in a structured questionnaire on their smoking habits, hypertension, diabetes dyslipidaemia, and any family history of coronary artery disease. Informed consent was obtained from all patients and controls, as required by our ethics committee.

Determination of risk factors: For CAD risk factors, the following definitions were used: subjects were defined as hypertensive if their blood pressure was > 140/90 mm Hg or if they were receiving any antihypertensive treatment; those with a history of diabetes or who were receiving any anti dia-betic drugs and fasting glucose levels > 120 mgs/dl were consi-dered to be diabetic; those with a total plasma cholesterol concen-tration of > 200 mgs/dl or a triglyceride concentration of> 180 mgs/dl, or who were receiving lipid lowering drugs were considered dyslipidemic. Smoking history was recorded as either none or current smokers. A positive family history was the presence of a first degree relative with coronary artery disease at the age of < 55 years for men and < 60 years for women.

Each participating inpatient with CAD was interviewed to determine variables, such as smoking, alcohol use, family history ($\geq$1 first-degree relatives with MI or coronary artery disease), and to obtain consent for a blood sample for genetic analysis. In addition, detailed chart abstractions were performed to collect relevant laboratory and clinical data. A total of 100 CAD patients included in the current study agreed to participate and to provide a blood sample for genetic analysis. Patients self-reported their race/ethnicity by selecting one of the following descriptors that were provided by the investigators: family history, diets, occupation, etc.

Protocol approval: The institutional review boards of both institutions approved the research protocol; all study participants were provided with written informed consent for clinical and genetic studies

Angiographic study: All patients underwent coronary angiography. Coronary stenosis was considered significant in the presence of a luminal diameter narrowing of > 50% of at least one pericardial coronary artery. The severity of coronary artery disease was expressed by the number of affected vessels (one, two, or three vessel disease) and also by means of the Duke scoring system13-a prognostic index that includes the number of diseased major vessels, the presence of left main coronary artery disease, the percentage narrowing of the major vessels, and involvement of the left anterior descending coro nary artery particularly when the proximal segment shows severe stenosis (> 95%). The Duke score ranges from 0–100 (0 = no disease, 100 = the most severe disease).
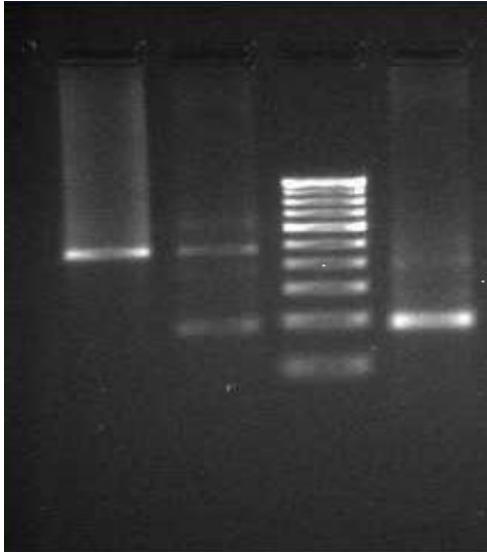
Figure 1. PCR analysis of ACE gene polymorphism. Lane 1, 490 bp (II); lane, 2- 490 and 190 bp (I/D); lane, 3 100 bp ladder; lane 4 190 bp (DD).

Biochemical analysis: Blood samples (5 ml) were collected from all subjects after 12 h fasting and some amount (2 ml) of collected samples were placed in EDTA tubes and stored at -80ºC until the time of assay for bio-chemical assays.

The serum concentrations of triglyceride (TG), total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C), urea, crea-tenine, fasting blood sugar (FBS) were measured by the standard methods (Auto-analyzer) used in the clinical laboratory of the hospital at the time of diagnosis of the patients.

Analysis of ACE gene polymorphisms

The ACE gene is located on chromosome 17q23 and consists of 26 exons and 25 introns. The intron 16 of this gene was the subject of this investigation. The primer sequences used for amplification were:

5_-CTGGAGACCACTCCCATCCTTTCT-3_ (forward primer), and
5_- GATGTCGCCATCACATTCGTCAGAT-3_ (reverse primer).

Extraction of DNA: Genomic DNA was extracted from whole fresh blood using standard phenol/chloroform methodology with ethanol precipitation. PCR was carried out using purified DNA. The ACE I and D alleles were identified by PCR amplification (Rigat et al., 1992).

The final concentration of the PCR mixture was 1.5 mM MgCl2, 50 mM KCl (Bio-Serve Hyderabad), 10 mM Tris-HCl (pH 8.8), 0.1% gelatin, 1% Triton X-100, 0.3 mM each of dNTPs (SIGMA), and 2U Taq DNA (bioserve Hyderabad) polymerase in each reaction tube. Amplification was carried out in a thermo cycler (TAKARA). The PCR was carried out under the following conditions: initial denatu-ration at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60^{0}C for 1.15 min, extension at 72°C for 2.30 min and final extension at 72°C for 5 min. The PCR products (Figure 1) were of 490 bp for allele I and 190 bp for allele D. The products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. In order to reduce mistyping of ID hete-

rozygotes as DD homozygotes, a re-amplification was carried out in all identified DD homozygotes using an internal primer specific for the I allele.

Statistical analysis

The means of the three-genotype groups (DD, ID, II) were com--pared in a one-way analysis of variance. We determined whether the distribution of the ACE genotypes was in Hardy-Weinberg equi-librium using chi-squared analysis, as described (Emery et al., 1976). For the ACE genotype, odds ratios were calculated as a measure of the association with the presence or absence of coro-ary artery disease. Comparisons of genotype and allele frequencies between cases and control subjects were performed by a χ 2 test with Fisher's correction. Statistical significance was accepted at $P <$ 0.05. Odds ratios (OR) as well as their 95% CI were computed to assess the strength of the association between the presence of the polymorphic alleles, genotypes, and CAD.

The analysis was also carried out by means of an explorative multiple logistic regression analysis to assess the independent role of the different factors possibly influencing the presence or absence of coronary artery disease, the extent of coronary artery disease. Dependent variables- age, sex, smoking, body mass index, dia-betes, hypertension, the plasma lipids and lipoproteins, and the ACE genotype are independent variables. All analyses were done using a personal computer with EPI 6 software (Epi info6 CDC).

Calculation of power analysis: Power analysis was done by using Power and Precision V3 soft-ware. Taking Alpha = 0.05 (2 tailed), and was found to be 0.96 with 100 as sample size.

RESULTS

Patients and risk factors

A total of 100 CAD patients were genotyped for ACE gene and were compared to controls (non CAD) popula-tion of 100 individuals. The P-values were calculated for individual categories and were observed for level of signi-ficance under p < 0.05. Demographic data of the popu-lation studied are shown in Table 1. Mean age was 57.21 years in patient group and 54.32 years in control group. Risk factor profile of CAD patients revealed hypertension in 34%; diabetes, 43%; smokers, 22%; and alcoholic, 26% patients and in controls hypertensive, 10; diabetics, 13; smokers, 8; and alcoholics, 10, respectively.

Classical risk factors

As expected, both the control and patient group showed differences in the biochemical markers and other conven-tional risk factors analyzed. Systolic and diastolic blood pressure, dyslipidaemia, arterial hypertension, diabetes mellitus, triglycerides and a previous record of CAD in the family are much higher in CAD patients than in controls. In average, glucose levels, triglycerides, LDL, createnine were also higher in the CAD group when compared to control group. We performed a multivariate logistic regression analysis using the variables as shown in Table 2. We found that the biochemical parameters like glyce-mia, CAD history, smoking habit, dyslipidaemia and ACE

Table 1. Demographic characteristics of patients and controls

	Cases n=100	**Controls n=100**	**p-value**
Age males(21-82)	57.21±14.05	54.32±24	0.81
Age females(20-72)	51.69±11.12	50.62±10.72	0.67
Alcoholism %	26	10	<0.01
Diabetics %	41	13	<0.001
Hypertension %	34	10	<0.01
Smokers %	37	8	<0.001

Values are represented as mean ± SD: p < 0.05 in comparison to control group.

Table 2. Biochemical characteristics of patients and controls.

Parameters	**Cases n=100**	**Controls n=100**	**p-value**
Total cholesterol	223.69 ± 62.32	203.34 ± 23.12	0.67
Triglycerides (mgs/dl)	203.34 ± 85.6	153.41 ± 17.81	<0.0001
HDL-C (mgs/dl)	45.56 ± 13.95	41.16 ± 10.16	0.15
LDL-C (mgs/dl)	136.79 ± 58.09	151.81 ± 63.08	< 0.001
CHO/HDL-C (mgs/dl)	5.18 ± 2.29	5.34 ± 1.17	0.45
LDL-C/HDL-C (mgs/dl)	3.18 ± 2.16	4.03 ± 2.14	<0.001
FBS (mgs/dl)	213.15 ± 118.21	124.72 ± 6.80	<0.0001
Urea (mgs/dl)	39.19 ± 14.10	35.18 ± 7.83	0.72
Createnine (mgs/dl)	1.16 ± 0.95	0.98 ± 1.12	<0.01

Values are represented as mean ± SD: p < 0.05 in comparison to control group.

I/D DD polymorphism to be independently related to CAD.

Genotype frequencies

The genotypic distribution of ACE gene polymorphism and allelic frequency for I and D alleles in patients and controls is given in Table 3. The ACE genotype Hardy-Weinberg equilibrium. The relative frequencies of ACE genotyping, that is, II and ID and DD in the controls and patient samples, did not differ significantly. When we determined the allele frequencies, it was found that the D allele was higher in patients as compared to controls (40% v. 28%). [x^2 = 5.20 (2df), p=0.07 for genotype; χ2 =6.46 (1df), p=0.01]. The ID genotype was 31% in patients while it was 23% in controls. The II genotype was a little higher in controls (61%) as compared to patients (45%); however, the differences were not significant. The odds ratio for ID genotype, DD genotype and D allele was 0.55 (95% CI; 0.27-1.12), 0.49 (95% CI; 0.22-1.10) and 0.58 (95% CI; 0.37-0.90).

The mean lipid levels, glucose, createnine levels of patients with CAD and controls were significantly different (Table 2). However, these levels were higher in patients as compared to controls. The ACE gene polymorphism has also been positively associated with triglycerides and low-density lipoprotein (LDL)-cholesterol, glucose levels, hypertension, diabetics, createnine levels, smokers and alcoholics.

DISCUSSION

For over a decade now, clinical and experimental researchers have hunted for gene polymorphisms as a major cause of hypertension, coronary artery disease distributions in both patients and controls were in the CAD) or heart failure, such as angiotensin-converting enzyme (ACE) polymorphisms, which were accused to be a major contributor of cardiovascular diseases (Cambien et al., 1992; Agerholm-Larsen et al., 2000). Sequence variants of the components of the renin-angiotensin-aldosterone system were suggested to have significant influences on cardiovascular homeostasis (Agerholm-Larsen et al., 2000; Schieffer et al., 2000). Our results suggest that the DD/ID genotype at the ACE gene locus might be an important genetic risk factor for CAD in familial hypercholesterolemia patients.

ACE is an attractive candidate gene to play a role in the vascular diseases including CAD. ACE gene plays important role in production of Angiotensin II and catabolism of bradykinin; involved in the modulation of vascular tone and the proliferation of smooth muscle cells. To our best knowledge this is the first study to investigate the association of ACE gene polymorphism and CAD in South Indian population. As the incidence of CAD is increasing in India, especially in the younger population, it is important to identify the risk factors for CAD. Our study does show significant difference in genotype and allelic frequency between CAD patients and healthy controls. The

Table 3. Distribution of ACE genotypes and allelic frequency of the study population.

Study Group	ACE Genotype				Allelic frequency		
	II	ID	DD	Total	I	D	Total
Patients (n)	45	31	24	100	121(0.60)	79 (0.40)	200
Control (n)	61	23	16	100	145 (0.72)	55 (0.28)	200

χ^2 =5.20(2df), p=0.074 for genotype; χ^2 =6.46(1df), p=0.01 for allelic frequency.

ACE gene is located on chromosome 17q23 and spans 21 kb on 26 exons. Insertion or absence of the 287-bp *Alu* sequence in intron 16 determines the *II, ID* and *DD* genotypes. Findings on the association between the ACE I/D polymorphism and atherosclerosis using genotyping have been inconsistent. Several studies have shown that the DD genotype is associated with a higher risk for myocardial infarction (MI) and CAD (Naftilan et al., 1989; Campbell-Boswell et al., 1981). Some studies showed a relation of atherosclerosis with presence of the D allele, (Castellano et al., 1995; Nergizoglu et al., 1999; Pujia et al., 1996; Hosoi et al., 1996) while others have failed to show such association (Diamantopoulos et al., 2002; Dessi-Fulgheri et al., 1995). The majority of the studies conducted until now were based on relatively small sample sizes, which may in part explain the inconsistency, particularly when interactions were studied (Lohmueller et al., 2003). A recent evaluation of candidate gene studies in a meta-analysis demonstrated that large studies are needed to show the effects of the genes involved in complex traits. The complexity of the results observed for the association of each polymorphism with CAD, together with the absence of a definitive functional gene variant, strongly suggests that future studies evaluating the effect of the ACE gene on cardiovascular disease should use a "gene based" approach. In addition, further and larger genetic association studies are needed in ethnic minorities with different allele/ genotype frequencies of the ACE variants and different patterns of cardiovascular disease.

The complex aetiology around cardiovascular disorders and the multiple environmental conditionings are most of the times not evaluated together. In most cases, CAD has a multifactorial genetic basis, involving a number of genes and environmental factors interacting to determine whether or not the disease will develop. Therefore, the inherited genes generally predispose to a greater or lesser extent of CAD, but it is the environmental factors (e.g. cigarette smoking, obesity, hypertension, sedentarism) interacting with the individual's genotype that determine whether or not CAD will develop. In the present study, we evaluated the association of the ACE gene with CAD, and found no association with the DD, ID, or II genotypes, but we found some statistically difference in allele frequency. However, in south Indian populations ACE gene polymorphisms seem to be a risk factor for stroke (Ajana et al., 2008). In a nutshell, our data do have

shown significant association between the ACE insertion/deletion polymorphism (D allele) and CAD in South Indian population which is somehow similar to the Vijay et al. study from south Indian population (Vijay et al., 2001).

Conclusion

The complex etiology around cardiovascular disorders and the multiple environmental conditionings are most of the times not evaluated together. In most cases, CAD has a multifactorial genetic basis, involving a number of genes and environmental factors interacting to determine whether or not the disease will develop. Therefore, the inherited genes generally predispose to a greater or lesser extent of CAD, but it is the environmental factors (e.g. cigarette smoking, obesity, hypertension, sedentarism) interacting with the individual's genotype that determine whether or not CAD will develop or make an individual susceptible to CAD. We performed further analysis in order to determine whether the simultaneous presence of genetic polymorphisms and well established risk factors–hypertension, smoking habit, obesity, diabetes and dyslipidaemia would enhance the effect in CAD onset. The vascular risk factors that might be related to serum ACE activity are not yet fully understood.

The above data suggest that the rate of the D alleles in association with certain demographic risk factors can contribute to the atherosclerosis. This kind of a statistically significant variation in the frequency of insertion /deletion in both populations strongly indicates that ACE polymorphism can pose a risk to CAD individuals. Our results also suggest that the D genotypes at the ACE gene locus might be an important genetic risk factor for CAD. The differences in the study of CAD among the various authors may result from specificity of population (regional environmental influences) or selection of study groups.

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