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Original Article

Angiotensin converting enzyme (I/D) and aldosterone synthase (-344T/C) gene polymorphisms as risks of coronary artery disease

Syed Tousif Ahmed[1*], Shamim Mushtaq[2*], Muhammad Israr Nasir[3*], Kanwal Fatima Aamir[4*], Pirzada Qasim Raza Siddiqui[1*]

[1]Department of Physiology, Ziauddin University, Karachi, Pakistan; [2]Department of Biochemistry, Ziauddin University, Karachi, Pakistan; [3]Department of Molecular Pathology, Fazaia Ruth Pfau Medical College, Karachi Campus, Air University Islamabad Pakistan, Pakistan; [4]National Institute of Cardiovascular Diseases, Karachi, Pakistan. [*]Equal contributors.

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Abstract: Objective: Coronary artery disease (CAD) is a leading cause of mortality and morbidity, globally, that involves genetic and environmental interaction with well-known risk factors, even in young individuals. The aim of this study was to analyze angiotensin converting enzyme (ACE) and aldosterone synthase (CYP11B2) genotypes in CAD patients and to study their association together with traditional risk factors. Methods: Case control study includes 239 participants aged 35-70 years, including 160 control and 79 patients with CAD who underwent coronary angiography. All subjects in the study were genotyped for ACE (II, DD) and CYP11B2 (CT, CC, TT) polymorphisms by polymerase chain reaction (PCR) in the genomic DNA. Data was analysed using SPSS 20 and *p*-value <0.05 was considered statistically significant. Results: Among the 79 CAD patients (mean age <50 years), 67.9% were male and 32.1% were female. Significantly higher levels of glucose fasting and random, TAG and Hba1c % were observed in CAD patients compared to control (P<0.001). The frequency of ACE II genotype was higher (33.3%) in CAD patients with OR 1.2 than in the control group (26.2%). However, CYP11B2 variant CT was found with high frequency (42.9%) in CAD patients with OR 0.8 (95% CI 0.3-2.3, P=0.79) compared to controls. No statistically significant differences were observed in the hyperlipidaemia and hypertension status between the two study groups. Conclusion: This study strengthens the association of CAD with CYP11B2 CT and ACE II genotypes in younger CAD patients with traditional risk factors.

Keywords: Coronary artery disease, angiotensin converting enzyme, CYP11B2 polymorphism

Introduction

Coronary artery disease (CAD) is a major cause of mortality in both low- and middle-income countries and accounts for nearly 7 million deaths [1-3]. The European Society of Cardiology guidelines [4] provides a diagnostic approach for managing patients with CAD. However, initial evaluation of patients starts with a history of comorbidities, symptoms (e.g. angina) and clinical investigations such as radiological evaluation, biochemical blood tests, ECG and coronary angiography. The gold standard for diagnosing CAD is coronary angiography (cardiac catheterization) which is an invasive technique. Risk factors of CAD includes hypertension, diabetes and dyslipidemia by both genetic and environmental factors [5, 6]. Conservative treatment is always the baseline treatment and it includes combining lifestyle-altering measures, such as exercise and diet, with cornerstone medical therapy [7]. In medical therapy, combinations of anti-ischemic drugs, nitrates, antiplatelet therapy and statin therapy are the most widely used. Invasive treatments of coronary artery disease (CAD) are coronary artery bypass operation (CABG) and percutaneous coronary intervention (PCI). After statin therapy, there is an approximate 13% mortality and relative risk has been reduced in patients after stroke, ischemic attack, myocardial infarction and even after CABG. Despite these treatments, the mortality in CAD is increasing globally which indicates the

need for detection of deeper molecular insights of CAD mechanisms and their polymorphic variants which could be therapeutic targets in the future. Several polymorphisms are important in the development and pathophysiology of CAD in some individuals but the list of variants associated with CAD is still expanding. The renin-angiotensin system (RAS) has an important role in regulating the blood pressure and pathophysiology of CAD. However, several studies have revealed that the polymorphic variants in the encoding components of the RAS are associated with CAD [8, 9].

Angiotensin converting enzyme (ACE) is a protease which regulates the RAS by cleaving the histidyl-leucine dipeptide from inactive angiotensin I (Ang I) and generates vasoconstrictor angiotensin II (Ang II). The Ang II exerts its function through receptors AngII receptor type 1 (AT1R) and AngII receptor type 2 (AT2R) whose action at the AT1R drives the synthesis and release of the aldosterone hormone. Moreover, aldosterone is a well-studied hormone which plays a role in the progression of cardiovascular diseases, such as hypertension and congestive heart failure [10].

Previous studies revealed that ACE gene polymorphisms such as insertions/deletions (I/D) are associated with various diseases such as myocardial infarction, coronary artery disease, hypertension and diabetes [11-13]. The regulation of aldosterone secretion occurs largely at the level of expression of the final enzyme required for its biosynthesis, aldosterone synthase (CYP11B2) which is a mitochondrial P450 oxidase key enzyme. One common single nucleotide polymorphism of the CYP11B2 gene is located in the promoter region [-344T>C with number of the ID: rs1799998] and has been associated with hypertension, heart failure, atrial fibrillation and atherosclerosis [14-16]. Despite previous studies, insufficient understanding of the genetic variants and susceptibility of CAD with association of traditional risk factors remains controversial. Therefore, we aim to analyze the relationship of ACE (II and DD) and CYP11B2 (-344T>C) genetic variations with other known risk factors and the possibility of CAD development.

Material and methods

Study groups

Our study subjects (n=239) consisted of 79 CAD patients and 160 controls (age; 35-70 years) admitted at the Ziauddin Hospital Clifton, and National Institute of Cardiovascular Disease (NICVD) Karachi, for diagnostic coronary angiography. The patients with unstable angina, old myocardial infarction and acute congestive heart failure were included in this study. Ethical approval (ERC Reference no-07/2019) was obtained for this case control study from the NICVD and Ethical committee of Ziauddin University. The 79 CAD cases were angiographically diagnosed with CAD and smoking status (consumption of 10 cigarettes per day at least for 3 months), hypertension, Diabetes (defined as hyperglycemia requiring antidiabetic drugs and/or fasting blood sugar (FBS) >126 and glucose tolerance test (GTT) >200) and BMI (Overweight: BMI, between 25-29.9) were noted. Control participants were volunteers recruited from the same hospitals, having regular check-ups and with no CAD history. Blood and fasting serum samples were collected for genotyping as well as assessment of glycaemic and lipid status. In the control group, a resting electrocardiogram was recorded to confirm the cardiac health status of all the subjects for this group. The study exclusion criteria were as follows 1) valvular heart disease, 2) cardiomyopathy, 3) cardiac hypertrophy, 4) myocarditis, 5) severe aortic stenosis, 6) refusal to take part in the study.

Genetic analysis

a) Isolation of genomic DNA: Isolation of genomic DNA was done from peripheral blood of CAD and control groups using a Diazole™ BD Reagent (Invitrogen™ PureLink™ Pro 96) according to manufacturer's instructions. The concentration and quality of DNA was checked using Qubit 2.0 Fluorimeter and Qubit dsDNA BR Assay Kit (Invitrogen™, Qubit™ dsDNA).

b) Polymerase chain reaction (PCR): PCR reaction was carried out in a volume of 25 µl with Accu Prime™ Taq DNA polymerase, 100 ng DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 5 mM dNTP mix and 10 µM each primer for analysis of CYP11B2, and ACE (I/D) gene polymorphisms. The genotyping of ACE (I/D) site could be seen by directly observing the amplification products of PCR. The primer sequence of two variants were summarized in **Table 1**.

c) The ACE gene was amplified using PCR mixture which was heated at 94°C for 5 min and underwent 35 cycles of amplification: denatur-

Table 1. The primer sequence of two variants

Gene	Forward primer sequence	Reverse primer sequence
ACE (rs1799752)	5'-CTGGAGACCACTCCCATCCTTTCT-3'	5'-GATGTGGCCATCACATTCGTCAGAT-3'
CYP11B2 (rs1799998)	5'-CAG GAG GAG ACC CCA TGT GAC-3'	5'-CCT CCA CCC TGT TCA GCC-3'

Table 2. Patient demographics between CAD and control groups

Parameters		Control N=160	CAD N=79	*p*-value
Age (years) Mean ± SD		48.04±8.26	49.69±9.18	0.225
Gender (n%)	Male	58 (69.0%)	57 (67.9%)	0.868
	Female	26 (31.0%)	27 (32.1%)	
BMI (kg/m^2) Mean ± SD		24.47±4.58	27.81±4.252	0.051
SBP (mmHg) Mean ± SD		121.47±5.87	125.16±17.96	0.075
DBP (mmHg) Mean ± SD		79.27±3.87	77.26±10.62	0.105
Smokers (n%)	Yes	14 (16.7%)	24 (28.6%)	0.065
	No	70 (83.3%)	60 (71.4%)	
Diabetes (n%)		0 (0.0%)	31 (36.9%)	<0.001

CAD: coronary artery disease; BMI: body mass index; DPM: diastolic blood pressure; SBM: systolic blood pressure.

ation 94°C for 30 s, annealing 58°C for 30 s, elongation 72°C for 30 s. The final elongation took 10 min at 72°C and the amplified product was analyzed on 2% agarose gel and stained with ethidium bromide. Amplified products gave rise to homozygous wild type II (one fragment of 490 pb) and homozygous mutant DD (one fragment of 190 bp) which were detected by ultraviolet transilluminator.

d) The CYP11B2 gene, was amplified using PCR conditions including a cycle at 95°C for 5 min, 35 cycles of denaturation at 94°C for 15 s, annealing at 67°C for 15 s, and elongation at 72°C for 5 min. The amplified fragments were digested with the Hae III restriction enzyme (Research Bio labs) and were subjected to electrophoresis on 2% ethidium bromide-stained agarose. The appeared fragments of 273 bp and 202 bp were detected by ultraviolet transilluminator.

Statistical analysis

Data was analyzed using SPSS 20. Categorical variables were expressed in frequency and compared by chi-square test between groups; quantitative variables were expressed in the form of mean ± standard deviation (SD) and compared by student-t test. Odds ratio (OR), confidence intervals (CI) 95% and *P* values were also calculated and significance was considered at *P* value less than 0.05. However, the allele frequencies were considered from the observed numbers of the genotypes and the chi-square test was used for the comparison in the genotypes between cases and control. P<0.05 was considered with statistical significance.

Results

Clinical and baseline characteristics of the study subjects

Seventy-nine (79) CAD patients and one hundred sixty (160) control subjects were examined.

The baseline characteristics of all the study subjects are summarized in **Table 2**. The mean age of the CAD patients and control subjects were 49.69±9.18 and 48.04±8.26 respectively (P=0.225). The sex distribution revealed that 57 CAD patients were males and 27 were females. The mean BMI values were significantly higher among CAD patients 27.81±4.252 and control subjects 24.47±4.58 (P=0.051). No significant difference in blood pressure (systolic and diastolic) was observed in CAD patients and control subjects. Regarding smokers, 16.7% smoked in the control group and 28.6% smoked in the CAD group, with an insignificant association noted between the groups, *p*-value 0.065.

Table 3. Biochemical parameters of CAD and control groups

Biochemical Parameter	Controls (N=160) Mean ± SD	CAD (N=79) Mean ± SD	*P* value
Creatinine	0.8±0.2	0.9±0.2	0.178
Urea mg/dl	17.6±2.4	22.5±8.8	<0.001
Mean FBS mg/dl	130.3±13.3	142.6±81.9	<0.001
Mean Glucose Random mg/dl	130.3±13.3	192.1±11.4	<0.001
Hba1c %	4.7±0.9%	6.9±1.3%	<0.001
Total Cholesterol mg/dl	169.1±10.06	174.9±20.9	<0.001
TAG mg/dl	136.8±25.8	154.2±29.7	<0.001
HDL mg/dl	43.9±3.8	40.6±5.0	<0.001
LDL mg/dl	97.7±11.8	103.04±21.7	<0.001
VLDL mg/dl	27.3±5.1	30.8±5.9	0.169

FBS: fasting blood sugar; TAG: triacylglyceride; HDL: high-density; LDL: low density lipoprotein; LDL: ow density lipoprotein; VLDL: very low density lipoprotein.

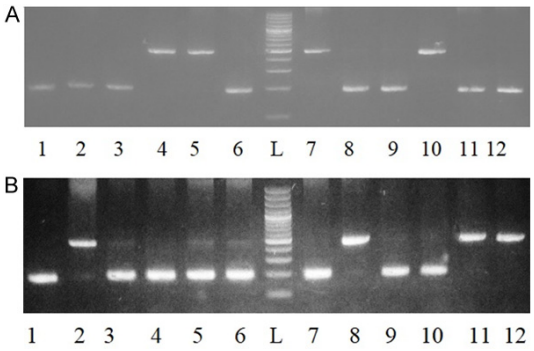

Figure 1. A. CAD cases: ACE genotyping (Deletion "D" allele or Insertion "I" alleles) were identified by the presence of 190 bp (Lane no. 1, 2, 3, 6, 8, 9, 11 & 12) and 490 bp fragments (lane no. 4, 5, 7 & 10). B. Control: 190 bp (Lane no. 1, 3, 4, 5, 6, 7, 9 & 10) and 490 bp fragments (lane no. 2, 8, 11 & 12). Lane (L) represents ladder 100 bp DNA.

The biochemical parameters are shown in **Table 3**. There were significantly higher levels of total cholesterol, triglycerides, FBS, glucose and HbA1c that were observed in CAD patients compared to control subjects. However, no significant difference was observed in urea, creatinine HDL, LDL and VLDL level among the CAD patients and controls.

Genotype distribution of ACE and CYP11B2G genes

The ACE II and DD genotypes were identified in CAD and control groups by the presence of 490 and 190 bp fragments (**Figure 1A** and **1B**). The frequency of II genotype was higher in CAD subjects than the control, 33.3% and 26.2% respectively. However, a significant high frequency of DD genotype was found in control 73.8% compared to CAD patients 66.7%. Significant association showed that the odds ratio (OR) of ACE II was 1.2 times higher in CAD patients with the DD variant (95% CI 0.6-2.1, P=0.8) as shown in **Table 4**.

The CYP11B2 gene polymorphism, (-344T) variant, is detected as fragments of 273 bp and the (-344C) variant as fragments of 202 bp (**Figure 2A** and **2B**). Being homozygous for C, heterozygous for C and T, or homozygous for T will be referred to as having the genotypes -344CC, -344CT, or -344TT, respectively. However, CT variant was found in high frequency (42.9%) in the CAD group compared to control with OR 1.2 (95% CI 0.3-4.4, P=0.79). It was found that the OR of developing CC variants was 0.9 times higher (95% CI 0.3-2.6, P=0.9) than TT variant 0.8 (95% CI 0.3-2.3, P=0.68) as shown in **Table 4**.

Discussion

Various genetic and environmental factors have an important role in the development of CAD and its risk factors may differ in each population. It is well proven that the interaction between genetic and environmental factors influences CAD onset and severity but it is difficult to explain which factors are relevant in development of the disease. However, limited research has been done on the genetic variations with traditional risk factors which could be responsible for the development of CAD. Thus, our study revealed that the ACE II, DD and

Table 4. Genotype frequencies of ACE and CYP11B2 genes

SNP		Control N=160	CAD N=79	OR	95% CI	*p*-value
ACE (rs1799752)	II	22 (26.2%)	28 (33.3%)	1.2	0.6-2.1	0.8
	DD	62 (73.8%)	56 (66.7%)	0.8	0.2-1.4	0.5
CYP11B2 (rs1799998)	CC	21 (25.0%)	20 (23.8%)	0.9	0.3-2.6	0.9
	CT	26 (31.0%)	36 (42.9%)	1.2	0.3-4.4	0.79
	TT	37 (44.0%)	28 (33.3%)	0.8	0.3-2.3	0.68

OR: odds ratio; 95% CI: 95% confidence interval.

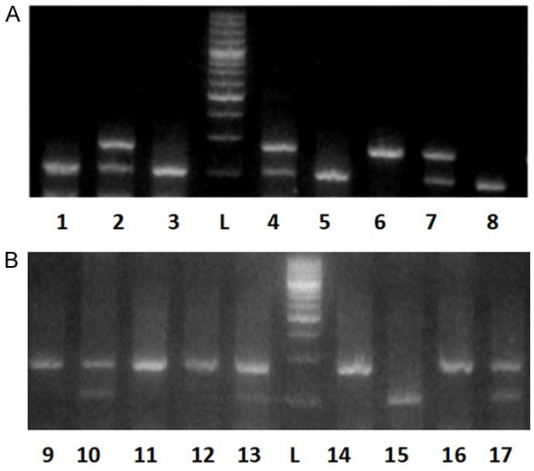

Figure 2. A. CAD cases: CYP11B2 genotyping (Thymidine Uncut the -344, "T" allele) was identified by the presence of 273 bp (Lane no. 6) and cut fragments cytosine "C" allele was identified by the presence of size of 202 bp (Lane no. 1, 3, 5, 8). Heterozygous CT genotype were identified by the presence of two bands at 273 bp and one band 202 bp (Lane no. 2, 4 & 7). B. Control: 273 bp (Lane no 9, 11, 12, 16) and 202 bp one band at (Lane no. 15). Heterozygous two bands at 273 bp and one band at 202 bp (Lane no. 10, 13, 17). Lane (L) represent ladder 100 bp DNA.

CYP11B2 (-344T & -344C) genotypes influence the development of CAD, interacting with conventional risk factors among the subset of the Karachi population.

CAD in younger individuals is becoming more challenging with its traditional risk factors during the last decade and considered a problem because of people's lifestyles and family history. We have also found CAD in study subjects with the average age of <50 years (49.69± 9.18) who were predominantly male (67.9%). However, a study on an Iranian population also revealed that five hundred and four (74.6%) of the CAD patients were male with the mean age of 60 years [16]. A Similar study in Pakistan on Kashmiri population showed that CAD patients were under the age of 45 years, who had hyperlipidemia, diabetes mellites and hypertension [17].

The present genotype study showed that high frequency of ACE II (33.3%) and CYP11B2 CT (42.9%) genotypes were found in CAD patients who had increased BMI, high level of total cholesterol, FBS, glucose random and HbA1c which are important factors for the development of CAD. However, a previous study reported that ACE I/D and CYP11B2 polymorphisms are associated with increased blood pressure that could increase the risk for the development of cardiovascular disease [14, 18]. A study on a Pakistani population revealed almost similar results and showed increased BMI with raised SBP, DBP and TC which clearly suggested a significant impact of BMI on the cardiovascular risk factors [19]. Conversely, our study showed insignificant results with SBP, DBP and TC between the CAD and control groups. However, a study on the Karachi population revealed a high prevalence of being overweight and obese as a clinical marker for CVD [20].

In the present study, the ACE DD variant was found to be associated with the control group rather than the genotype II in CAD patients. On the other hand, Guney et al. and his coworkers reported a more close association with CAD and the ACE D allele than the other two genotypes (ID and II) [21]. Moreover, a study done on the Iranian population showed no association of the ACE I/D polymorphism with risk of CAD [16]. Ethnic genetic differences could explain this disparity among the different populations.

Interestingly, there was a large meta-analysis done in 2122 patients with CAD and 1565 healthy controls to study the association bet-

ween CYP11B2 gene -344T>C polymorphism and CAD risk. Because of the difference in the genetic background and among different population environments, they have found no significant association between the CYP11B2 gene -344T>C polymorphism and CAD risk. However, the same study using an ethnicity-specific subgroup strategy, found a significant association between the CYP11B2 gene -344T>C polymorphism and CAD risk in both Caucasian and Asian populations [22].

Jandeleit Dahm et al. have reported that ACE significantly contributes to the pathogenesis of type 2 Diabetes and renin angiotensin aldosterone system inhibitors were shown to improve insulin resistance [23]. However, CYP11B2 -344T>C and ACE I/D polymorphism was also associated with type 2 Diabetes in European and Saudi populations [24, 25]. Our findings showed that the ACE II and CYP11B2 CT genotype is a marker mutation that deliberates the risk of CAD with diabetes (36.9%) as risk factor. However, the sample size was small to demonstrate the strong association of these genotype with CAD.

We have also found that the majority of non-smokers (71.4%) in the CAD group did not show any significant association with the control (83.3%) group. Previous studies have shown that cigarette smoking was found to be an important risk factor in Turkish CAD population [26, 27] and suggested that ACE expression increases with nicotine use and the D allele is found to be associated with endothelial dysfunction [22].

The present study had some limitations, our study group was not very large, and the sample size might have been too small to show the association of variants with well-established risk factors (such as lipidemia or hypertension) however, this does not disprove the key findings of the study. Future research with larger sample sizes with family history is required, which could focus on both younger (<50 years) and older CAD (>50 years) patients to reveal more comprehensive data which might contribute to better elucidation of the contribution of ACE and CYP11B2 genotypes.

Conclusion

The present case-control study strengthens the hypothesis that ACE II and CYP11B2 CT gene polymorphism are associated with CAD and are interacting with other risk factors such as total cholesterol, FBS, glucose, BMI and HbA1c. This raises the debate whether these variants are useful clinical markers to predict the risk of CAD in individuals.

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Informed consent was obtained from all of the patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Syed Tousif Ahmed, Department of Physiology, Ziauddin University, 4/B, Shahrah-e-Ghalib, Block 6, Clifton Karachi, Pakistan. Tel: +92-21-35862937-9; E-mail: tousif.ahmed@zu.edu.pk

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