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Original Article

Genetics Section

Association of Angiotensin Converting Enzyme Gene Insertion / Deletion Polymorphism with Risk of Ischemic Heart Disease in A Population of Smokers in Southern India

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ABSTRACT

Background: Ischemic heart disease (IHD) remains a major public health problem nationally and internationally. Smoking is a major risk factor for IHD.The deletion (D) allele of the angiotensin converting enzyme (ACE) gene polymorphism has been associated with hypertension, ischemic stroke and myocardial infarction. The present study was carried out to determine the association of the ACE gene insertion/deletion (I/D) polymorphism in IHD patients with and without smoking.

Materials and Methods: One hundred seven male IHD patients admitted consecutively in the Cardiology unit of a Government Hospital and 100 age and sex matched healthy controls were enrolled in this study.The patients were further divided into smokers and nonsmokers. All the subjects were checked for I/D polymorphism of ACE gene, which is mapped to 17q23.3 with OMIM no 106180, by polymerase chain reaction (PCR). The subjects were also investigated for lipid profile and ejection fraction (EF).

Results: We found significant difference in the distribution of D allele between patients and controls (p=0.009, OR 1.69, 95% CI 1.139 to 2.517).The significantly lower EF (p<0.001) was suggestive of greater cardiovascular compromise in smokers. The frequency of ID genotype was significantly associated with cases compared to controls (p=0.012, OR 2.054, 95% CI 1.1694-3.624) but was not significantly associated with smokers as compared to nonsmokers.

Conclusion: We infer significant association of D allele with IHD. The smokers with ID genotype should be put on prophylactic ACE inhibitor therapy to prevent the morbidity and mortality associated with IHD.

Keywords: Gene polymorphism, Smoking

INTRODUCTION

Ischemic heart disease has become a major public health problem in many developing countries including India. The prevalence of IHD has increased in India from 9.7% in 1995 among the urban population to 11.6% in 2009 [1]. It is conventionally said to be influenced by factors such as hypertension and smoking. The aetiopathogenesis of IHD suggests complex interaction between genetic factors and environmental risk factors [2]. According to Beegom et al., cigarette smoking increases the risk of IHD by a factor of 3.2 [3].Cigarette smoke contains toxic active molecules such as aldehydes, heavy metals, hydrogen cyanide and aromatic polycyclic hydrocarbons, which are responsible for excess oxidative stress leading to atherosclerosis and IHD [4-6].

Renin-angiotensin- aldosterone system (RAAS) plays a pivotal role in regulating the blood pressure. ACE is a zinc metallopeptidase that converts the inactive decapeptide angiotensin I to the active octapeptide and potent vasoconstrictor angiotensin II, which is the main active product of RAAS [7]. The gene encoding ACE has been mapped to 17q23.3. Rigat et al., found a polymorphism involving the insertion or deletion (I/D) of a 287-bp Alu sequence of DNA in intron 16 of the gene that influences the levels of the circulating enzyme [8]. Angiotensin converting enzyme gene is an attractive candidate gene, since it regulates ACE activity, which has been implicated in arterial wall myo-intimal proliferation in humans. Earlier studies reported D allele to be significantly associated with hypertension and atherosclerosis [9]. Schut et al., Sayed-Tabatabaei et al., and Munshi et al., have evaluated the association of I/D polymorphism of ACE with hypertension, stroke and coronary atherosclerosis [10-12]. However, there are very few reports on the role of conventional risk factor like smoking and ACE gene polymorphism on IHD from different regions of the world and especially from India [13,14].

The correlation of ACE gene polymorphism with smoking and its complex interaction with pathogenesis of IHD still remains controversial [15,16]. Thus, a comparative study between the smokers and non smokers suffering from IHD in a particular race or ethnicity could be highly useful in elucidating genetic differences in the pathogenesis of IHD and there by establishing suitable biomarkers for early monitoring, avoiding risk factors and for better management of IHD. The ACE gene polymorphism study can also help in pharmacogenomic applications as a cost effective tool for avoiding unnecessary trials and failures.

In the present study, we investigated the association of ACE gene I/D polymorphism and IHD between smokers and non-smokers of the cosmopolitan city of Hyderabad. The genotypic distribution and allelic frequencies were compared between IHD cases and controls and then in smokers and non-smokers. In addition to this, parameters of hypertension, ejection fraction (EF) as a measure of left ventricular contractility and lipid profile was evaluated in smoking and non-smoking patients of IHD as well as in age matched healthy controls.

MATERIALS AND METHODS

A total of 207 subjects were enrolled for our study, out of which 107 were consecutively admitted male patients with IHD, aged between 30-65 years, in the Intensive Cardiac Care Unit of Cardiology Department (ICCU, Gandhi Medical College and

Hospital, Secunderabad, India) over the period of September 2012 to November 2013 and 100 age matched healthy male controls. Seven patients could not be included in the analysis as they did not meet our selection criteria. The patients were subsequently categorized as smokers and non-smokers based on the history of smoking. (>/= 20 pack years of smoking i. e. 20 cigarettes per day for one year constitutes one pack year) [17].

All the patients were diagnosed with IHD according to the American College of Cardiology Foundation (ACCF) diagnostic criteria: chest pain lasting for > 3 h, ECG changes (ST elevation > 2mm in at least two leads) and elevation of enzymatic activity of serum creatine phosphokinase and aspartate aminotransferase [18]. Clinical parameters like Blood pressure (BP), height, weight, co-morbid condition and age of onset of IHD were documented in a well designed proforma. Patients with renal disease, hepatic disease and any other neurological disorders were excluded from the study.

The study was approved by institutional ethics committee. Informed consent was obtained from all the patients or relatives before collection of blood sample. Ten ml of blood was collected from each of these patients, for carrying out biochemical and molecular investigations.

Body mass index (BMI)

The body mass index of all the subjects was calculated by the accepted formula weight (kg)/height (meter)^2 [19].

Blood pressure

The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded by sphygmomanometer first thing in the morning on the next day of the admission, prior to collection of blood sample. Mean arterial blood pressure (MABP) was calculated with the formula: Diastolic pressure+1/3rd of Pulse pressure (DBP+1/3rdPP).

Left ventricular contractility (Ejection fraction)

2D echocardiography was done by an experienced cardiologist for all the patients and ejection fraction (EF) was recorded.

Lipid profile

Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low density lipoprotein (LDL) were done by auto-analyser (Hitachi 912). Very low density lipoprotein (VLDL) was calculated by Friedewald´s formula [20,21].

Genomic DNA extraction and genotyping

Genomic DNA was extracted from the EDTA blood samples using standard salting out method [22,23]. The purity of DNA was checked by nano drop. The polymorphism in ACE gene was detected using standard PCR technique. The primers used for the amplification of the intron 16(I/D) polymorphism and the method used has been published elsewhere [24]. The amplified PCR products were visualised on a gel documentation system after staining with ethidium bromide in 2% agarose gel after electrophoresis.The PCR conditions are given in [Table/Fig-1].

PCR Primers	Annealing temperature	Extension time	Primer Concentration	Total no of cycles
Forward: 5' ACCACTCCCATCCTTTCT-3'	59°C - 45 secs	72°C - 45 secs	Stock - 100 picomoles/µl	35
Reverse: 5' CCATCACATTCGTCAGAT-3'	Final Extension: 72°C - 5 mins		Working - 10 picomoles/µl	

[Table/Fig-1]: Standard PCR Conditions

STATISTICAL ANALYSIS

Hardy–Weinberg equilibrium was tested for the ACE gene polymorphism. Comparison of clinical parameters and lipid profile was done by t-test. Allelic frequencies were calculated according to the number of different alleles observed and the total number of alleles examined. Odds ratio with 95% confidence interval (CI) was calculated and genotypes association was done by Chi-square analysis using Open EPI6 software (Open Epi Version 2.3.1 from Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA). p-value of <0.001 was considered to be significant.

RESULTS

In the present study, out of 100 patients, who met with the inclusion criteria, 40 were smokers and 60 nonsmokers. The clinical details, cardiac parameters and the lipid profile are given in [Table/Fig-2]. Genotyping was done for 200 subjects, which included 100 healthy controls. The frequencies of genotypes and alleles are given in [Table/Fig-3].

Variables	Nonsmokers (n=60)	Smokers (n=40)	p-value
Age(yrs)	56.11 ± 5.81	50.47 ± 5.13	<0.001
BMI(kg/m^2)	18.92 ± 1.44	20.44 ± 2.04	<0.001
SBP (mm of Hg)	127.9± 12.99	128.74 ± 15.5	NS
DBP (mm of Hg)	80.27 ± 4.54	78.61 ± 5.31	NS
MABP (mm of Hg)	95.98 ± 6.2	94.94 ± 7.07	NS
Ejection fraction (%)	64.55 ± 3.61	47.56 ± 4.24	<0.001
TC(mg/dl)	183.30 ± 15.60	242.82 ± 35.52	<0.001
HDL(mg/dl)	45.64 ± 6.91	35.58 ± 6.26	<0.001
LDL(mg/dl)	100.69 ± 9.80	167.23 ± 41.91	<0.001
VLDL(mg/dl)	22.80 ± 5.78	32.03 ± 3.77	<0.001
TG(mg/dl)	135.06 ± 26.46	160.30 ± 18.58	<0.001
TC/HDL ratio	4.15±3.86	7.09± 2.12	<0.001
LDL/HDL ratio	2.41±1.38	4.79±1.53	<0.001

[Table/Fig-2]: Comparison of clinical parameters and lipid profile in smokers with IHD and non-smokers with IHD (data expressed as mean ± SD)

Genotype	Patients	Controls
II	10	32
ID	64	46
DD	26	22
Alleles	**Patients**	**Controls**
I	84	110
D	116	90

[Table/Fig-3]: Genotypes and alleles of IHD patients and controls

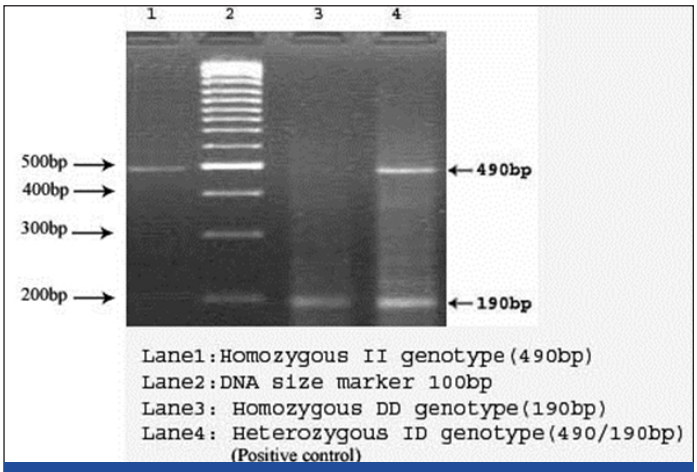
The mean age at diagnosis of IHD was significantly lower (p<0.001) in smokers as compared to nonsmokers. The body mass index of smokers was significantly higher compared to non smokers (p<0.001). There was no significant difference observed in SBP, DBP and MABP among both the groups. The ejection fraction of smokers was significantly lower than non-smokers (p<0.001).

TC, LDL, VLDL and TG were significantly higher, whereas HDL significantly lower in the group of smokers. The TC/HDL and the LDL/HDL ratios were significantly higher in the smokers (p<0.001).

The PCR product of ACE gene was 190 base pair fragment in case of deletion (D allele), and a 490 base pair fragment in the presence of insertion (I allele). Thus, there were three genotypes after electrophoresis: a 490 bp band (II), a 190 bp band (DD), or heterozygous 490 and 190bp bands (ID), [Table/Fig-4].

The D allele was significantly (p<0.009) associated with IHD (OR 1.69, 95%CI 1.139 to 2.517) when compared to I alleles [Table/Fig-5]. The data followed dominant and codominant models of inheritance with ID showing a two fold increase and the ID+DD a

fourfold increase in risk [Table/Fig-5]. When the IHD cases were categorized as smokers and non smokers, it was seen that although the D allele frequency was more in smokers it was not statistically significant [Table/Fig-6].



[Table/Fig-4]: 2% Agarose gel picture showing PCR products for ACE gene polymorphism

Genotypes	OR	p-value	95%confidence interval
ID + DD vs II	4.1882	0.0003	1.9255 to 9.1100
ID vs DD+II	2.054	0.012	1.1694 to 3.624
DD vs II+ID	1.262	0.482	0.6853 to 2.422
D allele	1.69	0.009	1.139 to 2.517

[Table/Fig-5]: Odds ratio of genotypes of IHD patients and controls

Genotypes	OR	p-value	95%confidence interval
ID + DD vs II	7.020	0.0700	0.8161 to 57.7885
ID vs DD+II	1.9385	0.1337	0.8161 to 2.2427
DD vs II+ID	0.8958	0.8142	0.3579 to 2.2427
D allele	1.015	0.950	0.6343 to 1.624

[Table/Fig-6]: Odds ratio of genotypes in smokers and non smokers

DISCUSSION

Smoking is a potent risk factor for cardiovascular morbidity and mortality. In IHD, the formation of an occlusive thrombus at the site of rupture of a plaque in the coronary arteries leads to reduced circulation to that part of the myocardium and compromises its contractility, eventually causing heart failure [18].

In the present study, we observed that the mean age at diagnosis of IHD is 50.47±5.13 yrs in smokers which is, significantly lower in comparison to non-smokers (p<0.001). Our study supports that of Panwar et al., who reported association of smoking with lower age of onset and higher mortality from IHD in Indian smokers [25]. To our surprise smokers had higher BMI than non smokers, which was contradictory to earlier studies by Viswanathan et al., [26]. However, Chieloro et al., made similar observations and have suggested that heavy smokers tend to have greater body weight as there is a likelyhood of clustering unhealthy life style in smokers (e.g.; low degree of physical activity, inappropriate diet, and alcohol) which is responsible for weight gain [27].

Hypertension is one of the relevant risk factors for IHD but there was no significant difference observed in SBP, DBP and MABP of both the groups which can be attributed to the fact that during the time of recording of blood pressure, both smoker as well as non smoker group were under similar anti-hypertensive therapy and were being continuously monitored for stable BP in the cardiac care unit. However EF, was significantly lower in smokers when compared to non-smokers (p<0.001) which explains the reduced left ventricular contractility owing to greater myocardial damage in smokers. Our findings are concurrent with that of Ambrose et al., who have also reported EF to be significantly lower in chronic smokers in comparison to healthy adults [6].

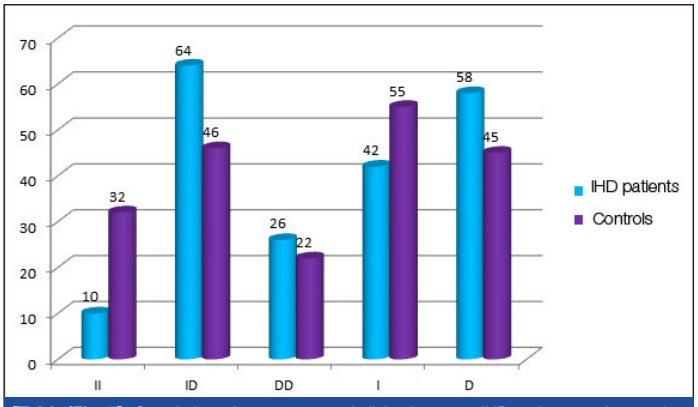
Dyslipidemia is another factor associated with IHD. In our study it was observed that the TC, LDL, VLDL, TG and the atherosclerotic indices were increased significantly [Table/Fig-3], while HDL was significantly decreased in smokers (p<0.001). Smoking causes an increased activity of HMG-CoA reductase and decreased lipoprotein lipase activities, resulting in elevated levels of TC and TGL, LDL, and VLDL [28]. In addition, nicotine stimulates the sympathetic adrenal system, which leads to increased lipolysis. According to McCall et al., the reduced HDL level in smokers is due to its increased catabolism and the inactivation of the lecithin-cholesterol acyl transferse (LCAT) system [29].

While analysing the association of mutant alleles with IHD, we observed that the D allele indeed plays a role in predisposing individuals to IHD (OR 1. 69, 95% CI 1.139 to 2.517, p=0.009) as compared to I allele.

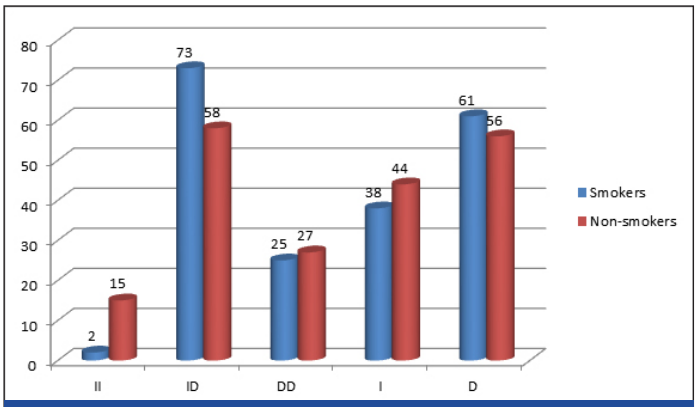
While comparing IHD patients and controls [Table/Fig-7], we observed a co-dominant model of inheritance to be associated with IHD (OR 2.054, 95% CI 1.1694 to 3.624, p=0.012). Earlier studies of Jamil et al., and Dhar et al., have reported the D allele of ACE gene to be significantly associated with morbidity in coronary artery disease in the South and East Indians [30,31]. However, while comparing smokers and non smokers, we observed that even though the frequency of D allele was much higher, it was not statistically significant in smokers (OR 1.015, 95% CI 0.634 to 1.624, p=0.950).

We observed a significantly higher occurrence of ID genotype (OR=2.054, CI=1.1694 to 3.624, p=0.012) among smokers [Table/Fig-8], suggesting that prophylactic therapy with ACE inhibitors may be appropriate for management or prevention of IHD in smokers. Our study supports the result of Kaur et al., who have reported a significant association of the ACE ID+DD genotype with 2.4-fold increased risk of acute myocardial infarction with smoking in a North Indian population [32].

Our findings support the results of Sayed-Tabatabaei et al., and Zak et al., who stated that the interaction between ACE ID polymorphism and smoking may be crucial in the cardiovascular mortality particularly at a younger age based on carotid intima media thickness [16,33].



[Table/Fig-7]: Correlation of genotypes and alleles between IHD patients and controls



[Table/Fig-8]: Genotype correlation between smokers and non smokers

In a similar study of an Egyptian population, Abdel-hamid et al., [14] reported the heterozygous "ID" genotype to be more prevalent among smokers suffering from IHD. Recently Manfrini et al., have stated that ACE inhibitor therapy seems to be an effective first line treatment for preventing mortality in patients with non-obstructive coronary artery disease [34].

LIMITATIONS

The limitations of this study are: (i) the relatively small sample size and (ii) our inability to incorporate the therapeutic data of patients which could have given us an idea about the response to treatment with ACE inhibitors in smokers and non-smokers.

CONCLUSION

Results of the present preliminary study along with evidence from literature enables us to suggest that individuals with a history of >/= 20 pack years of smoking irrespective of their BMI, SBP, DBP, TG, VLDL and HDL, should be monitored for MABP, TC, LDL and HDL/LDL ratio and evaluated for ACE I/D polymorphism to stratify them as high risk for development of IHD. Those smokers with ID genotype should be put on prophylactic ACE inhibitor therapy to prevent the morbidity and mortality associated with IHD.

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