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Study of Angiotensin Converting Enzyme gene insertion/deletion polymorphism, rs4340 in an urban Indian cohort, South Mumbai

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Running Head: ACE 'I/D' polymorphism in Indian Cohort

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Abstract

The insertion/deletion polymorphism (rs4340) of the Angiotensin Converting Enzyme (ACE) gene has been considered as a genetic risk factor for cardiovascular disease. The present cross sectional study aimed to evaluate the ACE gene insertion/deletion polymorphism in a cohort of urban residents from Mumbai, India. A total of 190 unrelated subjects, aged 15-93 years, attending Bay View Clinic participated in the study. Metabolic variables including blood pressure and blood glucose levels were measured. The 'I/D' polymorphism, rs4340 was determined by polymerase chain reaction, PCR followed by agarose gel electrophoresis. The 'I/D' genotype in heterozygotes was confirmed by a second PCR using insertion-specific primers.

Among the study cohort, 70 subjects (36.8%) had metabolic disorders.The allele frequency was found to be as 0.61 for the 'I' allele and 0.39 for the 'D' allele. The distribution of I/I, I/D and D/D genotype was 37.4%, 47.9% and 14.7%, respectively. People in the age group of 46-55 years had high distribution of 'I/I' genotype (52.4%). Study subjectsin the younger age group of 15-24 years had the highest frequency of 21.8% for the 'D/D' genotype, considered as a high risk genotype. In the present study, 'D/D' genotype was associated with higher body mass index and lower age even in the absence of established cardiovascular risk factors. Obesity was more prevalent in women (66.6%) as compared to men (33.4%). In conclusion, we found 'I/D' heterozygosity of the polymorphism,rs4340 as the most common genotype among this hospital cohort, with a slight over representation of 'D' allele among obese and overweight subjects.

Key-words: ACE, Mumbai, Gene

Introduction

Cardiovascular disease (CVD) is the major cause of mortality in the world [1]. An estimated 17.7 million people died of CVD in 2015, representing 31% of all global deaths [2]. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke [3]. Mortality due to cardiovascular diseases in India increased from 1.3 million in 1990 to 2.8 million in 2018 [2]. According to World Health Organization (WHO), it is predicted that by 2030, 23.6 million deaths will be due to cardiovascular diseases worldwide [3].

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Established risk factors for cardiovascular disease include unhealthy diet, physical inactivity, alcohol, tobacco use or smoking [3]. Multiple other risk factors such as obesity, hypertension diabetes and dyslipidemiaalso contribute to the pathogenesis of cardiovascular disease. Blood pressure is controlled by a signaling pathway, known as Renin Angiotensin Aldosterone System, RAAS[4].When renal blood flow or blood pressure is reduced, prorenin from the kidney is converted into renin and is released into the blood. Renin carries out the conversion of circulating angiotensinogen, a protein secreted by the liver, to Angiotensin I. Angiotensin converting enzyme (ACE) via the passage of lungs, causes the conversion of decapeptide, Angiotensin I to octapeptide, Angiotensin II, a potent vasoconstrictor [4].

One of the best studied genetic modifications in ACE gene is the insertion/ deletion (I/D) polymorphism(rs4340). This polymorphism was identified by Rigat et al in 1990 [5]. There is an insertion or deletion of a 287 bp sequence, within the Alu region, in the intron 16 of the ACE gene [5]. Studies suggest that deletion allele denoted as 'D' is associated with higher plasma levels of ACE activity and increased conversion of Angiotensin I to Angiotensin II. Angiotensin Converting Enzyme levels have been found to be intermediate in individuals having 'I/D' genotype and least in individuals with 'I/I' genotype. Further the 'D' allele has been reported to be associated with cardiovascular diseases. This I/D polymorphism affects 47% of ACEenzyme activity in blood [5].

There are limited cohort studies involving Indian subjects. The present study aims to investigate ACE 'I/D' polymorphism (rs4340) in a hospital cohort, from south Mumbai, western India. Although ACE 'I/D' polymorphism has been studied previously, to our knowledge this is the first study of an urban cohort attending a hospital in Mumbai, western India.

Material and Methods

Study population

Subjects who underwent routine blood test at the pathology department of the Bay View Clinic, during 24 June 2019 to 31 October 2019 were enrolled for the study. The study subjects were unrelated upto first cousin. Patients with clinical history of tuberculosis and cancer were excluded from the study. Detailed characteristics of subjects which included age, sex, weight, height, smoking status, alcohol history, medical history and use of medicines were recorded with the help of a structured questionnaire.Blood glucose levels were recorded from the patient's medical case papers of the clinic. Blood pressure was measured using an automatic blood pressure monitor (Omron Hem 7121, Japan). The study was approved by the Human Ethics Committee of Bay View Clinic, Mumbai.

Blood sampling

Blood (2.0-4.0 ml each) was collected in vacutainers coated with Ethylene Diamine Tetra Acetate, EDTA and plain vacutainers. Serumwas separated by centrifuging the blood samples collected in plain vacutainers and dispensed into micro centrifuge tubes which was used for biochemical analysis. Serum cholesterol and High-Sensitive C-reactive protein assay (hs-CRP) were performed by enzymatic methods.

DNA extraction and Determination of ACE 'I/D' genotype

Peripheral blood DNA was isolated by a modified salting out method based on the original method of Miller et al [6], with changes suggested by Maurya et al [7]. The ACE I/D genotype was carried out by the method of Lindpainter et al [8].The ACE-forward and ACE-reverse primerswere5'-GCCCTGCAGGTGTCTGCAGCATGT-3'and5'GGATGGCTCTCCCCGCCTTGTCTC-3', respectively. Reactions were set up in a volume of 25 µl containing 7.5 µl H2O, 12.5 µl PCR master mix (Taq DNA polymerase, MgCl2 and dNTPs), 0.5 µl of of each primer, and 4 µl of deoxyribonucleic acid (DNA). The thermocycling procedure (Bio-rad Laboratories; Model number: MyCycler^{TM}Thermal Cycler) consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 45 seconds, elongation at 72°C for 2 minutes, and a final extension at 72°C for 7 min.The PCR products were separated on 1.5 % agarose gel containing 1.5µl ethidiumbromide and 7.0 µl PCR product. This method yielded amplification products of 597 base pairs(bp) for the I/I genotype, 319 bp for the D/D genotype and 597 bp + 319 bp for the I/D

Genotype. In case of heterozygous samples, a heteroduplex DNA product was more commonly seen near 597 bp region.

The D allele in heterozygous samples is preferentially amplified, thus each sample with homozygous'DD' genotype was subjected to a second round of PCR amplification using a set of insertion- specific primers; hace5a, 5'-TGGGACCACAGCGCCCGCCACTAC-3' and hace5c, 5'-TCGCCAGCCCTCCCATGCCCATAA-3', under the same PCR condition except for an annealing temperature of 67°C. Only the I allele yielded a 335 bp amplicon in this PCR reaction.

Statistical analysis:

Values of body mass index (BMI), blood pressure, age, blood glucose levels are expressed as Mean ± SD. Body mass index (BMI) was calculated by weight (kg)/height squared (m^2) Statistical significance of differences between two groups were assessed by unpaired student 't' test. A value of $p < 0.05$ was considered to be statistically significant. The observed frequencies were compared with expected frequenciesand tested for Hardy Weinbergequilibrium(HWE significance level atp<0.05). Difference in genotype frequencies between groups were tested by Pearson's chi square (χ^2) test.

Results and Discussion

The study project involves the analysis of an insertion/deletion polymorphism, rs4340 in the gene encoding angiotensin converting enzyme. A total of 190 subjects attending the hospital participated in the study. Table 1 describes the characteristics of study subjects. The subjects were aged 15-93 years (mean 49 years) and 59.0% were men. Subjects with history of hypertension were 26 (13.7%); subjects with diabetes were 14 (7.4%) and subjects with both hypertension and diabetes were 21 (11.0%). One hundred and twenty subjects were free from these metabolic disorders. Subjects with clinical history were under treatment.

Allele and genotype distribution in the study subjects: Allele frequency in this study cohort was found to be 0.61 for the 'I' allele and 0.39 for the 'D' allele. Allele frequencies of 'I' allele were found to be 0.61 and 0.60 for men and women, respectively. Similarly there was no difference in the distribution of the 'D' allele between men and women. The distribution of various genotypes of I/I, I/D and D/D were 37.4%, 47.9% and 14.7%, respectively.

Table 1: Characteristics of study subjects

VARIABLES	
Total Number Of Subjects	190
Men (%)	112 (59.0 %)
Women (%)	78 (41.0 %)
Age (Mean ± SD) years	48.9 ± 20.2
Body Mass Index (Mean ± SD) Kg/m²	23.2 ± 5.5
Blood Pressure (Mean ± SD) mmHg	
Systolic	119.0 ± 21.4
Diastolic	72.6 ± 11.9
Clinical History – number (%)	
Hypertension only	26 (13.7%)
Diabetes only	14 (7.4%)
Hypertension and Diabetes	21 (11.0%)
Coronary Artery Bypass Graft	2 (1.0%)
High Cholesterol	7 (3.7%)
Number Treated (%)	
Hypertension	45 (95.8%)
Diabetes (oral)	33 (89.2%)
(insulin)	2 (5.4%)
High cholesterol	6 (85.8%)
Subjects With No Clinical History	120 (63.2%)

Table 2: Age-wise distribution of genotype

	GENOTYPE			
Age group (years)	**I/I n (%)**	**I/D n (%)**	**D/D n(%)**	**Total n = 190**
15-24	6 (26.0%)	12 (52.2%)	5 (21.8%)	23
25-35	16 (38.1%)	20 (47.7%)	6 (14.2%)	42
36-45	11 (35.5%)	15 (48.4 %)	5 (16.1%)	31
46-55	11 (52.4%)	9 (42.9%)	1 (4.7%)	21
56-65	10 (41.7%)	11 (45.8%)	3 (12.5%)	24
65 +	17 (34.7%)	24 (49.0%)	8 (16.3 %)	49

Table 2 describes the age-wise distribution of genotype. People in the age group of 46-55 years had high distribution of 'I/I' genotype (52.4%). Frequency of 'I/I' genotype was found to be least in younger subjects aged 15-24 years (26.0%).

In the present study, we investigated the frequency of a common polymorphism, rs4340 involved in the renin angiotensin aldosterone pathway, in a small cohort of urban residents from Mumbai, India. While the study is ongoing, preliminary findings of the study are:

1. About one third of the subjects were suffering from various metabolic disorders. Isolated hypertension was present in 13.7% of the subjects while type 2 diabetes with and without hypertension, was present in18.4% of the subjects.
2. The frequency of 'D/D' genotype was highest in age group of 15-24 years (21.8%) and lowest in people aged 46-55 years (4.7%).
3. A significant proportion of subjects (26.0%) were obese according to the WHO criterion for the Indian population. Obesity was more prevalent among women (66.6%) compared to men (33.4%). The allele frequency among the obese subjects was found to be 0.5 for both 'I' and 'D' allele. Subjects with homozygous 'D/D' genotype had higher body mass index compared to subjects with other genotypes.
4. In this hospital cohort, the high risk genotype 'D/D' of rs4340, was associated with higher body mass index and lower age even in the absence of established cardiovascular risk factors.

The present study subjects are a small cohort of urban residents from Mumbai, Western India. About one third (36.8%) of this cohort were suffering from various lifestyle disorders. Hypertension was prevalent at a relatively younger age of around 35 years. A study involving an urban cohort from Delhi, North India reported a higher annual incidence of hypertension and diabetes at a younger age [9]. High prevalence of traditional risk factors seen in our cohort could play a role in increased cardiovascular mortality of Indians as reported previously [1].

In our study cohort, the frequency of 'I/D' genotype was found out to be more common (47.9%) compared to other genotypes. This finding is in agreement with a cohort study carried out in north India [10]. The 'D' allele of ACE 'I/D' polymorphism has been considered as a risk factor for cardiovascular diseases. The 'D' allele frequency of 0.39 found in our cohort is comparable to the 'D' allele frequency reported for other Indian ethnic groups such as Sikhs (0.51), Jat (0.45), Dogras (0.40), Assamese (0.40) and Kumaonese (0.39) [11]. The 'D' allele is reported to reach a frequency of 0.66 in India and 0.75 worldwide [10]. Study subjects in the age group of 46-55 years had high distribution of 'I/I' genotype (52.4%) while the youngest age group of 15-24 years registered high distribution of 'D/D' genotype (Table no 2).

In the present study, subjects with no clinical history had a frequency of about 15.6% for the homozygous 'D/D' genotype. These results are similar to the findings involving healthy south Indian population [12]. A report involving Mexican population reported a frequency of 24.0% for 'D/D' genotype in normal subjects [13]. Another case control study involving North

American population reported a frequency of 30% for the 'D' allele amongst the healthy control subjects [8].

The risk of obesity increased in subjects who carried one copy of the high risk 'D' allele. This finding is similar to the reports of Kabbany et al who found high frequency of the 'D' allele in Eyptian obese adolescents, of both the sexes, compared to healthy subjects [14]. In contrast to this Motawi and colleagues found no association between ACE 'I/D' polymorphism and obesity in Egyptian women [15].

This preliminary study provides a crude estimate of cardiovascular risk factors such as hypertension, diabetes and obesity in a cross section of urban Indians from Mumbai, Maharashtra. Standardized questionnaire, collection of data on blood pressure and other metabolic variables are the main strengths of the study. Since this is a hospital based study follow up investigations on the possible role of ACE polymorphism, rs4340 in the pathogenesis of future cardiovascular events would not be very difficult.This study has few limitations. As this study involves a hospital cohort, genotype and allele frequency of the study subjects may differ from the data derived from epidemiological studies. Our study involves a small sample size and the results need to be confirmed using a large sample size. This could eventually provide a better understanding of the association between the ACE I/D polymorphism and cardiovascular risk in our population.

The difference in the ACE polymorphism between populations may be due to differences in the genetic background arising from genetic drift. The present cross sectional study suggests that I/D polymorphism in the ACE gene can serve as a useful potential link for risk factors associated with cardiovascular disease. Future studies involving large number of subjects are needed to validate current findings.

Conclusion

In conclusion this preliminary study reveals the 'I/D' genotype of rs4340 as the most common genotype among the subjects, with a slight over representation of 'D' allele among obese and overweight subjects.

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