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Gender Specific Association of Insertion/Deletion Polymorphism of the Human Angiotensin Converting Enzyme Gene with Essential Hypertension

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ABSTRACT The pattern of angiotensin-converting enzyme *(ACE)* gene insertion/deletion (*I/D*) polymorphism in the Indian population is poorly known. The population has cultural and linguistic differences and lived in an environment that varied significantly from one region to another. There is controversy regarding the association of the Angiotensin-converting enzyme insertion/deletion polymorphism with essential hypertension and variation in blood pressure. In the present study we examined the importance of *ACE* (I/D) polymorphism as a determinant of hypertension and to assess the potential modifying effect of gender on ACE gene in Indian population. The *ACE* I/D polymorphism was assayed by PCR amplification of *ACE* gene in 200 hypertension patients and 200 controls. The genotypic and allelic frequencies were observed to be deviated significantly from Hardey-Weinberg equilibrium ($p<0.05$). The DD and ID genotypes were found to be strongly associated with hypertension in men with an odds ratio 2.25 (95% confidence level (CI), 1.14 to 4.42) and 2.20 (95% CI, 1.27 to 3.80) respectively, ($p<0.01$). Further, a linear relationship was observed between diastolic pressure and allele D in men but not in women. The data thus provide evidence in favor of an association of I/D polymorphism at the *ACE* locus (17q23) with essential hypertension. Moreover, these results highlight the potential importance of gender-dependent interactions between genetic background and expression of hypertensive phenotype. It further prompts the need for the confirmatory studies in large population-based samples.

INTRODUCTION

Hypertension and diabetes are known to be the important risk factors for cardiovascular and renal diseases. The relationship of the rennin–angiotensin system with essential hypertension (EHT) has been studied extensively over many years, but firm conclusions about a causal role have not been forthcoming (Laragh et al. 1990). The biologically active effector hormone of the system, Angiotensin II, is produced in the blood stream and involving rennin, secreted by the kidney, and angiotensin 1- converting enzyme (*ACE*), a membrane bound Dipeptidyl 1- Carboxy Peptidase ecto enzyme (encoded by the gene DCP1, also known as *ACE*) located in the endothelial lining of blood vessels through out the body (Erdos et al. 1987).

ACE is a Zinc metallo protease containing two functional domains. It catalyses the conversion of angiotensin 1 to the physiologically active peptide angiotensin II, which controls fluid electrolyte balance and systemic blood pressure (Ward et al. 1995) and in the modulation of vascular tone and in the proliferation of smooth muscle cells (Cardillo et al. 1999). Because of its key function in the renin–angiotensin system, many association studies have been performed with *ACE*. *ACE* gene has 26 exons and spans 21 Kb on chromosome *17q23* (Mattei et al. 1989). *ACE* gene has two promoters (Hubert et al. 1991). One of which is Somatic Promoter present at 5' side of first exon, and the other present at 5' side of germinal specific testicular *ACE* mRNA. The two promoters have their own cell specificities. The Somatic Promoter is active in endothelial, epithelial and neuronal cells and the germinal promoter is stage specific and active in male germinal cells (Howard et al. 1990).

Though the human *ACE* gene contains a number of variable polymorphic regions that can be of potential use in genetic analysis of populations (Reider et al. 1999), the *insertion/deletion (I/D) polymorphism* present in the *intron 16*, in particular has been extensively investigated (Howard et al. 1990). I/D

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polymorphism consists of the presence (Insertion- I) or absence (Deletion -D-) of *287 bp Alu repeat* element in intron 16 resulting in 3 genotypes viz, Insertion homozygote (I/I), Insertion/Deletion heterozygote (I/D) and deletion homozygote (D/D).

Association of ACE I/D polymorphism with essential hypertension (Mastana et al. 1997), myocardial infarction (Cambein et al. 1992), coronary heart disease (Lindpainter et al. 1995), development of nephropathy in insulin and non-insulin dependent diabetes mellitus (Vijay et al .2001) and diabetes mellitus (Lee et al. 2002) have been reported. It may also be a potential genetic marker in hypertensives at risk for renal complications (Pontremoli et al. 2000). Recently investigators have observed a potential influence of *ACE* gene polymorphism on fibrinolysis or endothelial function, (Butler et al .1999; George et al. 2001).

In the present study we have made an attempt to examine the importance of I/D polymorphism as a determinant of hypertension and also to assess the extent to which the association is modified by the sex/gender.

MATERIAL AND METHODS

The study was carried out on 200 (146 males, 54 females) hypertension patients, reported at CARE Hospital, - A Cardiac Center & Research Institute. Information regarding blood pressure and other clinical characteristics was obtained at study entry. Data were collected from each patient on clinical variables including age, height, weight, body mass index (Weight in kilograms divided by height in meters squared), cigarette smoking, alcohol consumption, family history and the presence of associated conditions like diabetes, ischemic heart disease along with antihypertensive drug therapy adopted. Diagnosis of hypertension was based on the physical and clinical examination of patients by the cardiologist followed by appropriate laboratory and other investigations.

Hypertension was defined as systolic blood pressure of >140 mm Hg or diastolic blood pressure of >90 mm Hg or current use of antihypertensive medication or if an individual had a history of hypertension (JNC 7^{th} report 2003). All the cases included in the study were of essential/primary type. Cases with secondary hypertension diagnosed on the basis of physical examination, biochemical and radiological investigations were excluded.

Simultaneously, 200 (147 males, 54 females) normotensive controls (BP<140/ 90 mm Hg) those volunteered to participate in the study were also examined for comparison with patient group. None of the control subjects were receiving antihypertensive therapy, treatment for heart disease or hormone replacement therapy at the time of investigation.

Determination of the ACE Genotype

DNA was isolated from whole blood using standard protocols (Miller et al. 1988; Lahiri et al. 1992) and *ACE* gene sequence was amplified by Polymerase Chain Reaction (PCR) according to protocol conditions and primer sequences published previously (Rigat et al. 1992). PCR products were detected on 2% agarose gel containing ethidium bromide. Deletion (D) allele was observed as 190 bp fragment and insertion (I) allele as 490 bp fragment. Individual DNA samples, genotyped as DD homozygotes, were subsequently analyzed by *complementary procedures* described else where (Shanmugan et al. 1993) to avoid *mistyping of DD homozygotes.*

Statistical Methods

Comparisons between case and control subjects of demographic variables, genotypes and allele frequencies were carried out by t test, ANOVA and χ^2-test respectively. Deviation from the Hardy Weinberg equilibrium was checked by a χ^2-test.

Because the prevalence of hypertension increases with age, all analyses were adjusted for age. The *age-adjusted* analyses were performed separately for men and women. The prevalence of hypertension was compared among the three *ACE* genotypes (reference group, II genotypes) with *multiple logistic regression* analysis. Analyses were performed with adjustment for other covariates (body mass index, family history, presence of associated conditions like diabetes and CAD), *Odds ratios* were calculated as a measure of the association of the *ACE* genotypes with the phenotype of hypertension with the effects of D allele assumed to be additive (with score of 0, 1, and 2 for II, ID and DD respectively), dominant (With scores of 0 for II and 1 for ID and DD combined) or recessive

(with score of 0 for II and ID combined and 1 for DD). For each odds ratio p values were calculated and *95% confidence intervals* were established. All statistical tests were carried out using the statistical package for the social sciences (SPSS10.0 version).

RESULTS

In the present study hypertensive men (146) and women (54) were compared with normotensive men (147) and women (53) for association with epidemiological factors as specified in table 1. It was observed that hypertension patients were slightly older than normotensive controls with mean levels of SBP and DBP being significantly higher in patients of both the sexes than in controls (P<0.01). Among men there was no difference in mean BMI values between the patients and control groups while hypertensive women were showing significantly increased mean BMI values as compared to controls (P<0.05). Hypertensive men and women showed statistically significant familial aggregation of hypertension (42.8% and 40.0%), when compared to control groups (19.1% and 15.2%). In both hypertensive and control groups greater involvement of genetic component was observed in Men. Prevalence of Diabetes Mellitus (DM) and Coronary Artery Disease (CAD) was observed to be greater in hypertensive men when compared to women. Similar comparison for the presence of DM and CAD was not possible in controls as they were selected without these conditions. When hypertensive men were compared to women, there were no significant differences with regard to age, BMI, SBP, DBP and family history.

Table 2 shows distribution of genotype and allele frequencies for *ACE* Insertion/deletion polymorphism. The overall frequencies of the genotypes II, ID and DD were 26.5%, 43.0% and

Table 1: Sex-wise distribution of base line characteristics observed in Hypertensive patients and normotensive control groups

Variable	*Men*		*Women*	
	HTN Cases (n=146)	*Control subjects (n=147)*	*HTN Cases (n=54)*	*Control subjects (n=53)*
Age, (mean,SE)	50.8(0.74)	46.0(0.62)	50.5(1.06)	44.5(1.05)
BMI, kg/m^2 (mean,SE)	26.3(0.31)	26.2(0.31)	26.4(0.56)	22.9(0.55)
SBP, mm Hg (mean,SE)	*150.8(1.81)	120.2(0.61)	*150.8(2.58)	119.1(0.81)
DBP, mm Hg (mean, SE)	*92.9(0.69)	80(0.29)	*91.2(0.98)	80.0(0.38)
Family history (%)	*42.8	19.1	*40.0	15.2
DM (%)	*31.7	Nil	29.1	0
CAD (%)	*23.5	Nil	*21.9	0

HTN- hypertension, SE- standard error, BMI- body mass index, SBP- systolic blood pressure, DBP- diastolic blood pressure, DM- diabetes mellitus, CAD- coronary heart disease.
Figures in parentheses represent standard errors.
* p<0.05 (χ^2-test, t-test,)

Table 2: ACE genotypes and allele frequencies in hypertensive patients and normotensive control groups

		Frequencies								*Total alleles on all chromosomes*	
		Genotypes						*Alleles*			
		II		*ID*		*DD*					
	n	*n*	*%*	*n*	*%*	*n*	*%*	*I*	*D*	*I*	*D*
HTN	200	53	26.5	86	43.0	61	30.5	0.48	0.52	192	208
CON	200	75	37.5	87	43.5	38	19.0	0.59	0.41	247	106

HTN- hypertension, CON- controls.
Genotype frequencies : χ^2=9.130, df 2, p<0.05.
Allele frequencies : χ^2 = 37.235, df 1, p<0.01.
Hardey-Weinberg Equilibrium : χ^2= 3.9326, df 1; P<0.05.

30.5% respectively in hypertensives and 37.5%, 43.5 and 19.0% in controls. The observed genotype frequencies were significantly deviating from the control group *(χ^2=9.130, df2, P<0.05*). The frequencies of allele D and I were *0.52 and 0.48* in patients while they were *0.41 and 0.59* in controls. The observed allelic frequencies in the hypertensive group differed significantly from those in the control group (χ^2=37.235. p<0.01 df1). Further the frequencies of alleles D and I were observed to deviate significantly from *Hardy Weinberg equilibrium* in patients (χ^2=3.9326 df 1; P<0.05) but not in the controls.

Table 3 shows the distribution of epidemiological factors by *ACE* genotypes. There were no significant differences among men across the three genotypes with regard to age and body mass index. In men consistent and statistically significant increase in age adjusted systolic and diastolic blood pressures with increasing number of D alleles was observed (P<0.01). The association was no longer statistically significant with multiple linear regression analysis. In hypertensive group men with DD homozygotes showed maximum frequency of familial history (52.2%) as compared to control men (46.7%). Presence of CAD and DM was more frequent in hypertensive men with DD homozygosity (45.7% and 45.7%).

Among hypertensive women II homozygotes were younger ($\bar{X}$ 48.3, SE 2.18) than allele D carriers ($\bar{X}$ 51.9, SE 1.89 and $\bar{X}$ 51.3, SE 1.60) and also hypertenisve men with II genotypes ($\bar{X}$ 51.7, SE 1.48). In contrast, in controls DD homozygotes were younger ($\bar{X}$ 41.4, SE 1.58) than those with other genotypes. In hypertensive women mean BMI values were reduced in II homozygotes ($\bar{X}$ 25.3, SE 0.88) as compared to other genotypes and also hypertensive men with II genotypes. Mean SBP levels were observed to be increasing with increase in the number of D alleles. In contrast, mean DBP values were decreasing with increase in the number of D alleles (Table 3). Higher incidence of hypertension was observed in the family members of female patients with DD genotypes (35.7%) as compared to ID and II (12.5% and 17.5%) genotypes. Further this difference was greater in hypertensive men with DD genotypes as compared to hypertensive women with DD genotype suggesting greater risk for men with family history of hypertension. It was also observed that among hypertensive women, patients with DD genotypes were at a greater risk for the development of associated conditions like CAD (14.3%) and DM (39.3%)

Table 3: Base line characteristics of men and women among hypertensives and normotensives by their *ACE* genotype

Characteristic	*ACE genotype (patients)*			*ACE genotype (controls)*		
	DD	*DI*	*II*	*DD*	*DI*	*II*
Men (n=146, 147)	46	61	39	28	56	63
Mean age, Y	50.7(1.29)	50.4(1.14)	51.7(1.48)	46.2(1.47)	45.0(0.92)	46.8(0.62)
Mean body mass index, kg/m2	26.8(0.58)	25.8(0.48)	26.6(0.53)	26.0(0.72)	26.9(0.57)	26.0(0.43)
Mean SBP mmHg	*156.1(3.17)	150.7(2.73)	144.1(4.4)	120.4(1.31)	120.54(1.03)	119.8(0.92)
Mean DBP mm Hg	*93.5(1.30)	92.8(1.05)	92.2(1.06)	79.6(0.63)	80.4(0.63)	79.8(0.28)
Family history, %	*52.2	34.4	43.6	46.7	32	42.9
CAD, %	*45.7	29.5	33.3	nil	nil	nil
DM, %	45.7	49.2	*69.2	nil	nil	nil
Smoking, %	*28.3	18	25.6	26.7	20	28.6
Alcohol,%	*37.0	26.2	33.3	20	28	35.7
Women (n=54,53)	15	25	14	10	31	12
Mean age	51.9(1.89)	51.3(1.60)	48.3(2.18)	41.4(1.58)	45.8(1.30)	43.6(2.84)
Mean body mass index, kg/m2	26.6(0.88)	26.9(0.99)	25.3(0.88)	21.2(1.05)	23.5(0.78)	22.8(1.00)
Mean SBP mmHg	153.4(5.85)	151.2(3.76)	146.4(4.52)	121(1.0)	118.5(1.15)	119.2(1.93)
Mean DBP mm Hg	88.1(1.77)	91.8(1.55)	93.6(1.69)	80(0)	80.0(0.66)	80.0(0.00)
Family history	*35.7	12.5	17.5	20.2	9.7	25
CAD %	*14.3	*14.3	9.5	0	0	0
DM %	*39.3	17.9	19	0	0	0
Smoking %	0	0	0	0	0	0
Alcohol %	0	0	0	0	0	0

*p<0.05

but less prevalent than hypertensive men (45.7% and 45.7% respectively).

The prevalence of hypertension according to *ACE* Genotypes was evaluated by logistic regression analysis. In men the age adjusted odds ratios (OR) for hypertension in the DD and ID groups were 2.25 (95% confidence interval (CI), 1.14 to 4.42) and 2.20 (95% CI 1.22 to 3.80) respectively, with the II genotype used as the reference group ($p<0.05$). In women there was no significant association of ACE genotype with hypertension, the adjusted Odds ratios being 1.20 (95 % CI, 0.38 to 3.92) and 0.44 (95 % CI, 0.17 to 1.06) respectively for the DD and ID groups ($p>0.05$).

In our study the frequencies of alleles D and I of *ACE* were more or less similar to those reported in other Indian populations (Pasha et al. 2002). We found consistent evidence, in men but not in women, of association of the ACE genotype with diastolic blood pressure and of association with hypertension. There was a slight increase in odds of hypertension with the ACE DD genotype, although there was no clear evidence of mode of mutation effect.

DISCUSSION

Heritable factors in combination with a number of recognized environmental risk factors are important determinants of the pathogenesis and natural history of essential hypertension. The notion that the presence of the allele D may identify ACE as one of the genes contributing to an increased risk of essential hypertension is both intriguing and provocative.

Previous work on Indian populations has shown a wide range of estimated frequencies from about 22 to 39 % for the II genotype, which is compatible with the 26.5% found in the present study (Pasha et al. 2002). In contrast estimated frequencies of DD genotype in our present study (30.5%), which shows a greater increase in the homozygotes, differed from reported population frequencies, (22%–27%) by Pasha et al. (2002). And also the allelic frequencies of hypertensive group (D 0.52, I 0.48) significantly deviate from normotensive group with high frequency of insertion allele (0.58, $p<0.05$).

Majumder et al. (1999) in a different context also reported higher frequency of *ACE* insertion allele in various ethnic groups. The higher frequency of I allele in the present study in control groups is in agreement with Asiatic and Mongoloid populations (Higashimori et al. 1993; Hong et al. 1997; Sagnella et al. 1999) but differs from the Americans, Caucasians and Europeans, who have a greater frequency of D allele and were reported to have a high risk of hypertension (Johanning et al. 1995; Morris 1996; Sagnella et al. 1999).

The association between the DD genotype and increased levels of plasma and tissue *ACE* (Rigat et al. 1990; Foy et al. 1996) provides a theoretical mechanism whereby the I/D polymorphism could be of potential importance in the control of blood pressure. There is a controversy regarding the association of the *ACE* locus with blood pressure and hypertension. In humans a positive association of the *ACE* D allele has been observed in some (Morise et al. 1994; Barley et al. 1996), but not in other case control studies of hypertension (Borecki et al. 1997; Fuentes et al. 2002). Our results demonstrated a difference in the distribution of the genotypes between patients and control groups as is evident from table 2. Among the patients there was increase in the number of DD homozygotes, which is consistent with a previous report (Mastana et al. 1997) from the Indian subcontinent claiming a significant association of DD genotype with hypertension.

Morise et al. (1994) found higher frequency of DD genotype among Japanese patients with hypertension compared with normotensives, more recently this has been confirmed in a large population study in Japanese men but not in women (Katsuya et al. 1998).

In our study which included 200 patients (146 men, 54 women) and 200 age and sex matched controls (147 men and 53 women), we observed a possible association of *ACE* D allele with hypertension in men but not in women, and also there was a linear relationship between diastolic blood pressure with D allele in men but not in women. This gender specific association is consistent and compatible with the Framingham study (O'Donnell et al. 1998). However this study is incompatible with a highly significant association reported between the D allele and hypertension in women of African descent (Sagnella et al. 1999).

In south Asians, no significant gender association between ACE I/D genotype and the prevalence of hypertension was shown by Sagnella et al. (1999). Our study provides

evidence that the effect of the *ACE* locus may be male specific. The hypothesis that there are sex differences in the effect of *ACE* I/D genotypes on blood pressure is supported by gene targeting experiments resulting in functional inactivation of the *ACE* gene in mice, in which the blood pressure effect predominates in males (Esther et al. 1996). Fornage et al. (1998) have recently reported that genetic variation in the region of the *ACE* gene significantly influences inter individual variation in blood pressure in men but not women.

In conclusion, our study shows a possible association of the *ACE* locus with hypertension and with blood pressure levels in men but not in women. The contradictory reports from several case control studies on the association of *ACE* locus with essential hypertension in various populations and multi-ethnic groups of Indian population supports the need for further confirmatory studies of association on large population based samples.

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