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Brief communication (Original)

Risk of angiotensin - converting enzyme (*ACE*) gene I/D and g.2350G>A polymorphisms in causing susceptibility to essential hypertension

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Background: Essential hypertension is a complex polygenic disorder arising from the interaction of several genes with environmental factors. Of the various physiological pathways affecting the homeostasis of blood pressure, the renin-angiotensin system (RAS) is considered important with angiotensin converting enzyme (*ACE*) playing a key role in causing susceptibility to hypertension.
***Objective*:** Explore the single locus, haplotype and epistasis patterns of two polymorphisms of *ACE* gene (I/D and g.2350G>A) and their contribution in causing risk for essential hypertension.
Methods: Two hundred seventy nine hypertensive cases and 200 healthy controls were recruited. Genotype of the *ACE* I/D polymorphism was determined by PCR and g.2350G>A polymorphism was assessed using a polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) based method.
Results: The distribution of genotypes showed a significant association of *ACE* I/D polymorphism ($\chi^2 = 7.148$, $p = 0.028$), while *ACE* g.2350G>A polymorphism did not show any such association ($\chi^2 = 0.85$, $p = 0.65$). For *ACE* I/D and g.2350G>A polymorphisms, genotypic proportions were statistically significant especially in males ($p < 0.05$). The frequency of H1 haplotype with *ACE* I and G2350 alleles was higher among hypertensives than in normotensives. The analysis for epistatic interaction showed a strong synergistic effect between I/D and g.2350G>A polymorphisms.
Conclusion: Our study suggests significant association of allele I of I/D polymorphism and essential hypertension with strong synergism when segregating with allele G of g.2350G>A polymorphism at *ACE* locus.

Keywords: Angiotensin-converting enzyme, essential hypertension, haplotype, I/D, g.2350G>A

Hypertension is one of the important risk factor for the development of coronary heart disease (CHD). It is also a common complex multifactorial disorder, caused by interplay of several candidate genes with additive effect and interactions with the environmental and demographic factors [1]. About 2.6 million Indians are estimated to die due to CHD alone by the year 2020 [2]. The renin-angiotensin system (RAS) plays a major regulatory role in the maintenance of blood pressure and electrolyte/blood volume homeostasis. Among the components of RAS, angiotensin 1- converting enzyme (*ACE*) has been extensively studied as a candidate biomarker in understanding the pathogenesis of hypertension.

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Angiotensin converting enzyme (*ACE*)-a monomeric membrane bound zinc-chloride dependent dipeptidyl carboxypeptidase I (DCP I; 3.4.15.1), is a bioactive component of renin angiotensin system (RAS) and kallikrein kinin system (KKS). Apart from the regulation of blood pressure and maintenance of fluid and electrolyte balance, the enzyme plays a significant role in the development of cardiovascular system and vascular remodeling. It activates angiotensin I (decapeptide) through cleavage of the carboxy terminal dipeptide into the potent vasoconstrictor angiotensin II and inactivates the vasodilator peptide bradykinin both of which act as mediators of vascular tone and smooth muscle cell proliferation [3].

The *ACE* gene is located on the long arm of chromosome 17q23, spans about 21 kb and comprises 26 exons and 25 introns [4]. The gene has two promoters 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 while the germinal promoter is stage specific and active in male germinal cells [5]. The levels of circulating *ACE* show extensive inter individual variability that is highly genetically determined [6]. An insertion/deletion (I/D) polymorphism, due to the presence or absence of a 287 base pair (bp) alu-type sequence in intron 16 of the *ACE* gene, has been shown to co-segregate with serum and tissue *ACE* activities. Individuals homozygous for the deletion are found to have approximately two-fold higher level of circulating enzyme as compared to homozygotes for the insertion [7].

Several studies have demonstrated association of *ACE* I/ D polymorphism with several cardiovascular diseases like cardiomyopathy, left ventricular hypertrophy, myocardial infarction, chronic renal diseases such as diabetic nephropathy and hypertension in various populations exhibiting both positive and negative associations [8-13]. Conflicting findings in various populations indicate involvement of environmental or ethnic factors as well.

Another polymorphism, *ACE* g.2350G>A lying in the exon 17 was found to exert 19% effect on plasma *ACE* concentration, and this polymorphism was shown to be in strong linkage disequilibrium with hypertension by Zhu et al (14). Further, g.2350G>A polymorphism was found to be associated with hypertension in the Emirati population and with acute myocardial infarction in Chinese Han population (15**)** and in Pakistan population [16].

In the present study we explored the single locus, haplotype and epistasis patterns of two *ACE* gene polymorphisms viz. I/D and g.2350G>A in determining susceptibility to essential hypertension.

Materials and methods

The present study includes 279 essential hypertensive patients and 200 age and sex matched normal, healthy individuals as control group. The patients studied were recruited at out patient clinic of Durgabai Deshmukh Hospital and Vijaya diagnostics centre and control samples were randomly collected from normal healthy population. The study was approved by the Departmental Ethical Committee. The blood samples and data were collected with informed consents from the participating subjects.

Data were collected from each patient on other variables including age, height, weight, body mass index (weight in kilograms divided by height in meters squared), history of cigarette smoking, alcohol consumption, familial incidence, and the presence of associated conditions like diabetes, CAD, and ischemic heart disease along with antihypertensive drugs advised. Subjects considered under essential hypertension group had systolic blood pressure of >140 and diastolic blood pressure of >90 mm Hg or those who were receiving antihypertensive therapy or medication at the time of investigation. All the cases included in the study were of primary type. Secondary forms of hypertension were ruled out based on clinical and laboratory investigations.

Five milliliters of blood was collected in an EDTA vaccutainer from patients and controls. Genomic DNA was isolated from peripheral blood lymphocytes using rapid non-enzymatic method [17].

Genotyping of ACE I/D polymorphism

The I/D polymorphism was assessed by detecting the presence (allele I, insertion) or absence (allele D, deletion) of a 287 bp in intron 16 of the *ACE* gene with the PCR technique by using the following oligos: sense 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and antisense 5'-GAT GTG GCC ATC ACA TTC GTC AGA T -3' [18]. PCR amplification of the genomic DNA was performed in a final volume of 10 □l reaction mixture containing ~100 -150 ng of DNA, 1 µl of 10X PCR buffer, 20 pmol/l of each primer, 200 µmol/l of each dNTPs, 0.25 U of Taq DNA polymerase and deionized water (varied). Each reaction mixture was carried out with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR products were resolved on 2% agarose gel and visualized following ethidium bromide (5 µg/µl) staining. The size of bands were estimated using 100 base pair ladder and the genotypes were determined as II with 490 bps, DD with 190 bps and ID with 490 and 190bps fragments. Since the D allele is preferentially amplified in heterozygous subjects, each sample found to be DD was reanalyzed by a second independent PCR amplification with an insertion-specific primer pair: sense 5'- TGG GAC CAC AGC GCC CGC CAC TAC - 3' and antisense 5' - TCG CCA GCC CTC

CCA TGC CCA TAA - 3' [19]. The reaction conditions and amplification parameters for this confirmatory reaction were the same as stated above. Known controls of each genotype were amplified with each set of samples analyzed for the *ACE* I/D polymorphism.

Genotyping of ACE g.2350G>A polymorphism

ACE gene codon 2350 sequence was amplified by PCR according to protocol conditions and primer sequences published previously by [20]. After amplification, 5 µl of the PCR product was digested with 5U of BstU1 enzyme (New England Biolabs, India**,** Catalog No.R0518) at 37°C overnight. Digested fragments were separated by electrophoresis on 3% agarose gel. The gel was stained with ethidium bromide (5 µg/µl) for 10 min and DNA fragments were visualized under UV transilluminator. *ACE* g.2350G >A genotypes were determined by the length of the fragments amplified. A fragment with 122 bps was identified as GG homozygote and with 100 and 22 bps as AA homozygote while appearance of all the three fragments was genotyped as GA heterozygote.

Data Analysis

Data collected was analyzed using SPSS package version 16.0. Continuous variables are expressed as means SD and the categorical variables as frequencies. An unpaired student's t-test was used to compare the difference between means of hypertensive cases and normotensive controls. Allele frequencies were calculated from genotype frequencies and difference in the distribution of genotype and allele frequencies between the cases and control groups were examined by χ^2 test. Odds ratios (OR) with 95% confidence intervals were estimated to calculate the relative risk for each genotype to develop the disease.

Haplotype analysis is a powerful tool for evaluating the alleles that mediate predisposition for a given condition. Haplotype analysis was done by using CHAPLIN statistical software [21]. We compared haplotype frequencies between hypertensives and normotensives using χ^2 test from a series of 2×2 contingency tables by combining other haplotypes.

Multifactor Dimensionality Reduction (MDR) analysis was performed using MDR software to determine the presence of epistatic interaction and the genotypic combination of the two genes that may confer high or low-risk for development of hypertension [22]. The analysis was implemented using open-source MDR software package (v.2.0.0) available from http://www.epistasis.org. In the present study, *ACE* I/D and g.2350G>A polymorphisms were analyzed by MDR for interaction analysis using the Relief F (RF) filter algorithm [23] and constructed possible combinations of single and two polymorphisms. Best models with possible combinations of the polymorphisms were considered based on 10-fold cross-validation and maximum testing accuracy.

Results

Demographic characteristics

The demographic and clinical details of the hypertensive and control subjects are presented in **Table 1**. The age of hypertensives ranged from 30 to 82 years and of controls ranged from 30 to 71 years. The mean levels of age, SBP, DBP and HDL-C in patients were significantly higher than in controls (p <0.05). The family history of hypertension and BMI among the patients was also statistically significant as compared to the controls, which suggest that genetic factors and body mass index can predispose individuals to hypertension. No significant differences were found between cases and controls for other risk factors such as the levels of total cholesterol, LDL-cholesterol, triglycerides, smoking habit and alcohol consumption (p >0.05).

Table 2 shows the distribution of genotypes of *ACE* I/D and g.2350G>A polymorphisms in hypertensives and normotensives. For *ACE* I/D polymorphism, the overall genotypic frequencies were deviated significantly between hypertensives and normotensives (χ^2 = 7.148, p = 0.028) showing an increased risk of hypertension for individuals with II genotypes (OR 1.64, 95% CI = 1.10-2.44) while individuals with ID genotypes were favored. The genotypic frequencies were in Hardy- Weinberg equilibrium in hypertensive patients while they were deviated in controls. The gender distribution of the *ACE* I/D and g.2350 G>A genotypes among hypertensives and controls are given in **Table 3**. The genotypic frequencies of *ACE* I/D polymorphism in male hypertensives deviated significantly when compared to male controls (χ^2 = 6.158, p = 0.046) indicating high risk of hypertension for males with II genotype. Regarding the g.2350G >A polymorphism

the genotypic distribution did not differ significantly between hypertensive patients and normotensive controls (χ^2 = 0.85, p = 0.65). However significant deviation was observed in hypertensive males as compared to normotensive males (χ^2 = 7.483, p = 0.023) indicating high risk for males with GG and AA homozygosity while heterozygote GA were protective against hypertension. The genotypic frequencies of g.2350G>A polymorphism was consistent with Hardy-Weinberg equilibrium in both hypertensives and controls (p >0.05).

Among other confounding factors the genotypic frequencies of the two polymorphisms deviated significantly between hypertensive patients who were used to alcohol consumption as compared with alcoholic controls (ACE I/D: χ^2 = 7.327, p = 0.026; g.2350G>A: χ^2 = 6.016, p = 0.049). The distribution of *ACE* I/D and g.2350G>A genotypes among sexes and in individuals with early onset (<45 years) and late onset (≥45 years) of hypertension are shown in **Table 4**. The frequency of *ACE* I/D and g.2350G>A genotypes tended to differ between the two age groups. However, this was not statistically significant (p >0.05). It is noteworthy that with the increase of age (late onset), increase in the frequency of II genotype of *ACE* I/D polymorphism (χ^2 = 4.708, p = 0.095) was significant at 10% level and GG and AA genotypes of g.2350G >A polymorphism (χ^2 = 6.220, p = 0.045) showed higher frequency that was statistically significant in male hypertensives as compared to normotensive males.

Table 1. Demographic and clinical characteristics of the study population.

	Patients (HT) n (%)	Controls (NT) n (%)	p-value*
Total	279	200	
Gender (M/F)	153:126	131: 69	
Age (years)	55.57±9.78	47.63±9.65	<0.0001
Age at onset	48.35±9.67	-	
BMI (Kg/m^2)	27.20±4.73	25.80±3.72	<0.0005
SBP (mmHg)	160.53±20.98	120.05±0.71	<0.0001
DBP (mmHg)	98.23±12.87	80.0 0.35	<0.0001
Alcoholic	63 (22.6)	46 (23)	0.914
Smoking	45 (16.1)	36 (18)	0.590
Family history	17 (64.2)	79 (39.5)	<0.001
TC (mmol/L)	181.9±48.06	190.9±83.17	0.229
LDL-C (mmol/L)	108.4±45.29	106.6±39.07	0.701
HDL-C (mmol/L)	39.3±8.58	41.3±8.89	0.042
TG (mmol/L)	178.3±104.52	167.3±79.98	0.296

HT = hypertensive patients, NT = normotensive controls. p <0.05*

Table 2. Percentage distribution of genotypes and alleles for ACE I/D and g.2350G>A polymorphisms in hypertensive cases and normotensive control subjects.

Gene polymorphism	Genotype	HT n (%)	NT n (%)	χ^2	p	OR (95%CI) HT vs. NT
ACE I/D	II	102 (36.6)	52 (26.0)	7.148*	0.028	1.64 (1.10-2.44, p=0.015)
	ID	127 (45.5)	114 (57.0)			0.63 (0.44-0.91, p=0.013)
	DD	50 (17.9)	34 (17.0)			1.06 (0.66-1.72, p=0.794)
Alleles	I	0.59	0.54	2.211	0.137	
	D	0.41	0.46			
g.2350G>A	GG	121 (43.4)	81 (40.5)	0.850	0.654	1.12 (0.78-1.63, p=0.531)
	GA	125 (44.8)	98 (49.0)			0.84 (0.59-1.22, p=0.364)
	AA	33 (11.8)	21 (10.5)			1.14 (0.64-2.04, p=0.650)
Alleles	G	0.66	0.65	0.601	0.80	
	A	0.34	0.35			

p <0.05*. Genotype frequencies are indicated as absolute values (values in parenthesis are percentages). Allele frequencies are indicated as fractions.

Table 3. The distribution of the ACE I/D and g.2350G>A genotypes between male and female among hypertensive and control subjects.

Genotype	Male			Female		
	HT n (%)	NT n (%)	χ^2	HT n (%)	NT n (%)	χ^2
ACE I/D						
II	61 (39.9)	35 (26.7)	6.158*	41 (32.5)	17 (24.6)	1.953
ID	65 (42.9)	73 (55.7)		62 (49.2)	41 (59.4)	
DD	27 (17.6)	23 (17.6)		23 (18.3)	11 (15.9)	
I	0.61	0.55	2.473	0.57	0.54	0.283
D	0.39	0.45		0.43	0.46	
g.2350G>A						
GG	69 (45.1)	49 (37.4)	7.483*	52 (41.3)	32 (46.4)	4.208
GA	60 (39.2)	71 (54.2)		65 (51.6)	27 (39.1)	
AA	24 (15.7)	11 (8.4)		9 (7.1)	10 (14.5)	
G	0.65	0.64	0.003	0.67	0.66	0.05
A	0.35	0.36		0.33	0.34	

Values in parenthesis are percentages; p<0.05*

Table 4. The frequencies of the *ACE* I/D and g.2350G>A genotypes by age, sex, and affection status.

Age group	Sex	Affection Status	n	*ACE* I/D genotype (frequencies)				g.2350G>A genotype (frequencies)			
				I/I	I/D	D/D	χ^2	GG	GA	AA	χ^2
<45	M	Hypertensive	20	0.40	0.55	0.10	1.384	0.50	0.45	0.05	0.8
		Normotensive	42	0.26	0.57	0.17		0.38	0.55	0.07	
	F	Hypertensive	11	0.09	0.91	0.00	2.162	0.36	0.67	0.00	2.967
		Normotensive	33	0.21	0.7	0.09		0.55	0.36	0.09	
	Total	Hypertensive	31	0.29	0.65	0.06	1.147	0.45	0.52	0.03	0.869
		Normotensive	75	0.24	0.63	0.13		0.45	0.47	0.08	
≥45	M	Hypertensive	133	0.40	0.41	0.19	4.708	0.44	0.38	0.17	6.220*
		Normotensive	89	0.27	0.55	0.18		0.37	0.54	0.09	
	F	Hypertensive	115	0.35	0.45	0.2	0.608	0.42	0.5	0.08	3.983
		Normotensive	36	0.28	0.50	0.22		0.39	0.42	0.19	
	Total	Hypertensive	248	0.38	0.43	0.19	4.538	0.43	0.44	0.13	1.422
		Normotensive	125	0.27	0.54	0.19		0.38	0.50	0.12	

p <0.05*

The distribution of haplotype frequencies in hypertensive cases and controls are shown in **Table 5**. Four haplotypes were evaluated in cases and controls and labeled as H1-H4. Though the individual haplotypes H1 (00), H2 (01), H3 (10), and H4 (11) did not differ significantly in their frequencies between patients and controls, the evaluated haplotype H1 (00), which contained *ACE* I allele and allele G of g.2350G>A occurred more frequently both in patients and controls with slightly higher frequency in the patient group.

Table 5. Haplotype frequency of the *ACE* I/D and g.2350G>A polymorphisms.

Haplotypes		Patients	Controls	OR (95%CI)	*p*-value
H1	00	306 (0.55)	204 (0.51)	1.171 (0.903-1.516)	0.2975
H2	01	22 (0.04)	12 (0.03)	1.328 (0.649-2.716)	0.1689
H3	10	60 (0.11)	54 (0.14)	0.772 (0.521-1.143)	0.2002
H4	11	164 (0.30)	126 (0.32)	0.905 (0.685-1.197)	0.3001

Common allele is indicated by 0 and rare allele as 1. The allele order is I/D and g.2350G>A from left to right. χ^2 (4x2) = 3.048, df = 3, p >0.05. Values in parenthesis indicate frequency of haplotypes.

Analysis of epistatic interaction

The present data included polymorphisms *ACE* I/D g.2350G>A as the over all best model with maximum testing accuracy of 0.530 and CV consistency of 10/10. High-risk (dark gray) and low-risk (light gray) genotypic combinations were determined based on the threshold value for the present data, which was 1.39 (279/200; **Figure 1**). It was observed that II genotype of *ACE* I/D when present in combination with AA genotype of G2350A showed a four-fold risk (4/1) while when present with GG a two-fold risk (90/45) for developing hypertension.

Figure 2 illustrates the MDR interaction information analysis of the two polymorphisms, represented in the form of a dendrogram. The interaction information analysis revealed a strong synergism between *ACE* gene polymorphisms I/D and g.2350G>A contributing to hypertension.

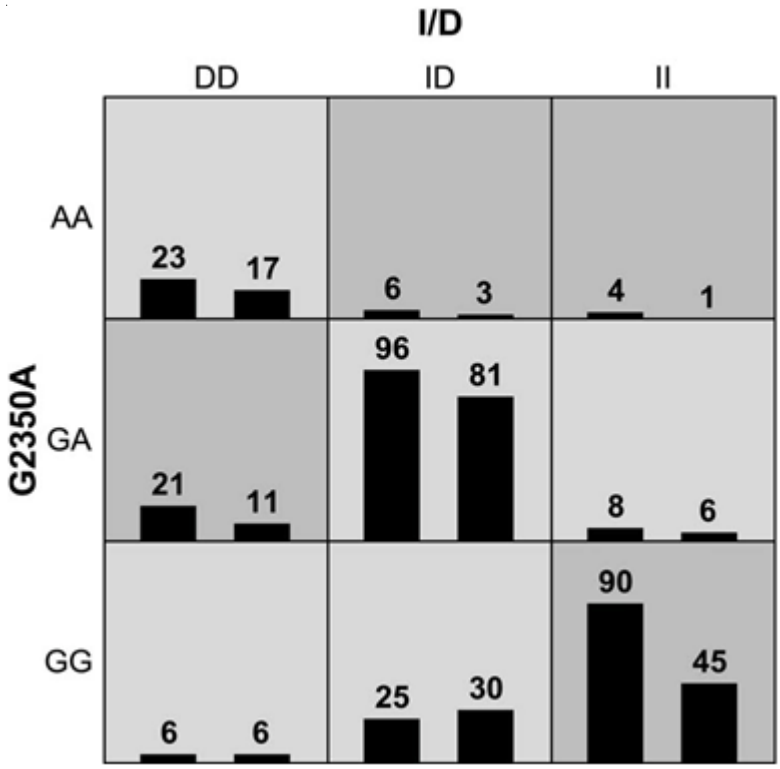

Figure 1. Distribution of high-risk (dark shaded) and low-risk (light shaded) genotypic combinations of the markers studied. The summary of the distribution illustrates the number of patients (left bars) and controls (right bars) for each genotype combinations.

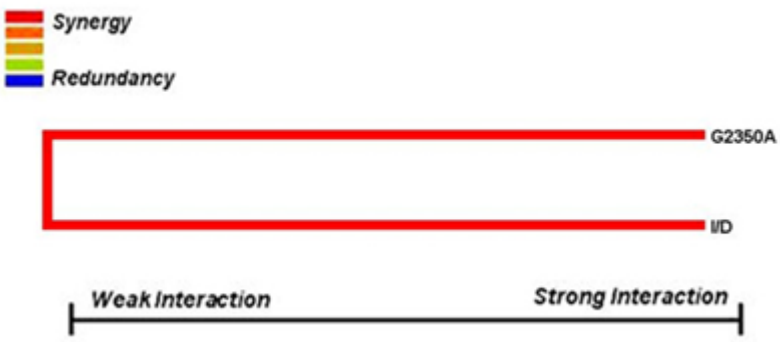

Figure 2. Interaction dendrogram for the two polymorphisms modeled by the MDR method that shows strong synergistic effect of the *ACE* I/D and g.2350G>A polymorphisms on hypertension development.

Discussion

Alarming increase has been observed over years regarding the incidence of hypertension in India and in other developing countries. It is estimated that the number of deaths in India due to cardiovascular disease would rise up to 4.6 million by the year 2020 and hypertension is directly responsible for 57% of stroke deaths and 24% of all coronary heart disease deaths [24]. Several studies suggested that *ACE* gene of RAS pathway that regulates blood pressure acts as a potential candidate gene for the study of hypertension and other cardiovascular diseases. The enzyme also plays a key role in the production of Angiotensin II and catabolism of bradykinin involved in the modulation of vascular tone and the proliferation of smooth muscle cells.

The present study is the first report investigating the association of *ACE* I/D together with G2350A polymorphism in causing susceptibility to hypertension from South India. There is a controversy regarding the association of the *ACE* polymorphism (I/D) with blood pressure and hypertension. Previous studies have reported a significant association of the *ACE* D allele with hypertension in African Americans, Chinese and Japanese population [25-27] while other studies have reported absence of such association [28-29]. In contrast to earlier findings allele I is reported to be associated with hypertension in the Australian and Pakistani populations [30-31]. The discrepancy between these studies in the distribution of *ACE* gene I/D polymorphism is explained as due to ethnic differences between the populations studied. In the present case control study, we found significant association of *ACE* II genotype with essential hypertension. The II genotype was higher in hypertensive cases (36.6%) as compared to controls (26.0%). Allele I was more common in males compared to females among hypertensive and control groups. Our study provides evidence that the effect of the *ACE* locus may be male specific. The observation 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 predominated in males [32].

A study by Sagnella et al [33] reported a higher frequency (0.40) of (II) genotype in expatriate South Asians in the UK. Similarly, a study from China [34] reported higher frequency (0.41) of II genotype. Previous work on Indian populations has shown a wide range of estimated frequencies from about 22 to 39 % for the II genotype [35], which is comparable with the 36.6% found in the present study. Several reports have shown that allele I is associated with insulin resistance, atrial fibrillation with hypertrophic cardiomyopathy, hypertension and mitral valve prolapse syndrome [36-39]. Thus, the *ACE* II genotype can act as a genetic risk factor for cardiovascular disease.

The *ACE* I/D polymorphic locus, identified in a non-coding sequence, is more likely to serve as a genetic marker because of its linkage disequilibrium (LD) with a putative disease causing locus located nearby. A study by Zhu et al [14] reported 13 polymorphisms in the *ACE* gene using linkage and association studies of which g.2350G>A polymorphism was accounting for 19% of the total variance in *ACE* plasma levels. After adjustment for the effect of *ACE* g.2350G>A dimorphism, the I/D polymorphism was not found to be associated with plasma *ACE* concentration, indicating that it is in LD with *ACE*

g.2350G>A polymorphism and unlikely to be a functional mutation. A study from Emirati population reported a significant association of g.2350G>A polymorphism and hypertension [20]. In contrast to their finding, our data did not show significant association between g.2350G>A dimorphism and essential hypertension but it shows significant association with male hypertensives as compared with controls. The proportion of GG genotype was found to be high in male hypertensive (45%) as compared to male control (37%). Among hypertensive patients, males have a higher percentage of GG genotype compared to female patients.

Further, individuals who were with II genotype of *ACE* I/D and GG and AA genotypes of g.2350G>A polymorphism with habit of alcoholic consumption were at risk evidenced by the high occurrence of hypertensives in these groups. In our study in the late onset group (≥45 years) a significant association of GG and AA homozygotes of g.2350G>A polymorphism was found between male hypertensives when compared with normotensive controls. However, a study by [31] showed a significant association between the *ACE* I/I genotype and young hypertensive individuals (below 40 years).

Haplotype construction revealed that among the possible haplotypes, 00 (I G) allelic combinations were the most frequent (OR 1.171) and 01 (I A) allelic combination was higher in the hypertensive group (OR 1.328). However, the combinations were not significant. Gene interaction studies give much more insight into the understanding of the complex genetic studies such as hypertension. Interaction analysis also revealed greater risk for II genotype of *ACE* I/D polymorphism in combination with AA or GG genotype of g.2350G>A polymorphism.

Conclusion

The present study suggests influence of I/D and g.2350G>A polymorphisms of *ACE* gene in the development of essential hypertension, especially in males.

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