

## ACE Gene Insertion/Deletion Polymorphism and Type-2 Diabetic Nephropathy in Eastern Indian Population

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### ABSTRACT

**Background:** Nephropathy is one of the major complications among the patients having type 1 or long term Type 2 diabetes and there are various studies that suggest its genetic predisposition. A 287 bp insertion/deletion (I/D) polymorphism of the gene encoding angiotensin-I converting enzyme (ACE) is shown to have association with diabetic nephropathy.

**Aim:** To identify the association of ACE I/D polymorphism with subjects having diabetic nephropathy.

**Materials and methods:** The present study examined the prevalence of ACE insertion/deletion polymorphism among 91 Bengali individuals from Eastern India. Among them 30 individuals belong to diabetic nephropathy (DN), 30 individuals having diabetes without nephropathy (DM) and 31 normal controls. The DNA samples of studied subjects were genotyped using polymerase chain reaction.

**Results:** The frequency of DD, ID and II genotypes in patients having diabetic nephropathy (DN) were found to be 26.7%, 53.3% and 20.0% respectively, whereas the same for only diabetic patients (DM) were 26.7%, 50.0% and 23.3% respectively. The frequencies of the same genotypes among the normal controls were found to be 9.68%, 64.5% and 25.8% respectively. In spite of a slightly higher odds ratio for DD genotypes among DM and DN subjects in comparison to the normal group the distribution pattern of DD genotype did not differ significantly within the three cohorts. The frequency of D allele among the patients having diabetic nephropathy, diabetic without nephropathy and control subjects was found to be 0.533, 0.516 and 0.420 respectively. This distribution pattern also did not differ significantly ( $\chi^2=1.859$ ,  $p>0.05$ ).

**Conclusion:** No significant association was found between ACE I/D polymorphism with diabetic nephropathy patients from Bengali caste population.

**Keywords:** Angiotensin converting enzyme, diabetic nephropathy, population variation.

### INTRODUCTION

Diabetes mellitus is a rapidly emerging global health problem and it is expected to cross the pandemic level within a span of another two decades. Worldwide its frequency is expected to rise from 171 million to 366 million within the next two decades and India alone will take almost 25% share from it to become the leading country with highest number of diabetic patients (Wild *et al.*, 2004).

Longstanding Type 2 diabetes mellitus sometimes leads to microvascular complications such as nephropathy, neuropathy and retinopathy. Among these complications nephropathy is the leading cause of chronic kidney disease in patients with diabetes mellitus and characterised

by albuminuria which leads to declining glomerular filtration rate (GFR) and ultimately damage the kidneys (Michel *et al.*, 2007). The prevalence of diabetic nephropathy in Type 2 diabetes subjects is reported to be 5-9% from various Indian studies (Acharya and Chowla, 1978; John and Kangasabapathy, 1991) and nearly 30% of chronic renal failures in India are due to diabetic nephropathy (Agarwal and Dash, 2000).

The Renin-Angiotensin-Aldosterone System (RAAS) is a regulator of both blood pressure and kidney functions and is suggested to play an important role in the development of nephropathy in Type 2 Diabetes Mellitus (Remuzzi *et al.*, 2008; Wang *et al.*, 2012; Felehgari *et al.*, 2012). Angiotensin converting enzyme (ACE) polymorphism is a major component of Renin-Angiotensin-Aldosterone System. The ACE gene is located on chromosome 17q23 consisting of 26 exons, 25 introns and it spans 21 kb. The polymorphism of ACE gene results from the insertion (I) or deletion (D) of a 287 bp *Alu* repeat sequence near the 3' end of intron 16 (Rigat *et al.*, 1992) which leads to three genotypes DD, II and ID. The mean plasma/serum ACE level in the DD subjects is reported to be approximately double than that of II subjects, with ID subjects having intermediate values (Rigat *et al.* 1990). The main function of ACE is the conversion of Angiotensin I to vasoactive, natriuretic octapeptide angiotensin II and is thus implicated in the pathogenesis of diabetic nephropathy (Jeffers *et al.*, 1997; Estacio *et al.*, 1998; Grzeszczak *et al.*, 1998; Bedir *et al.*, 1999; Viswanathan *et al.*, 2001). However there are studies which suggest no association between the etiologies of diabetic nephropathy with ACE genotype polymorphism (Kumar *et al.*, 2005). Therefore with the present state of knowledge the genotype-phenotype interaction between ACE gene polymorphism and diabetic nephropathy have not yet been fully understood. An extensive interethnic variation in the distribution of ACE gene polymorphism appears to be one of the causes for such inconsistent findings (Barley *et al.*, 1994; Jayapalan *et al.*, 2008). Therefore, the aim of the present study was to investigate the distribution of ACE gene polymorphism and its relationship with Type 2 Diabetic Nephropathy patients in Eastern Indian population as no such genetic data is available from this part of India inspite of a high prevalence of diabetic nephropathy cases.

## **MATERIALS AND METHODS**

The present study on ACE gene polymorphism was carried out among 91 age sex matched subjects belonging to Bengali caste population from Kolkata. The subjects include 30 age-matched Type 2 Diabetes patients without nephropathy (DM), 30 Type 2 Diabetes patients with Nephropathy (DN) and 31 healthy controls from Kolkata city.

Registered patients were recruited from B.P. Poddar Hospital and Research Centre, Kolkata as well as Calcutta Medical College and Hospital, Kolkata and a detailed medical history of each patient was recorded accordingly. The detection of Type 2 diabetic as well as diabetic nephropathic patients was based on physician's recommendation. Patient's age greater than or equal to 50 years and duration of type 2 diabetes greater than or equal to 5 years were the inclusion criteria for type 2 diabetes subjects. Selection of patients with diabetic nephropathy were based on the same age range with that of diabetic patients and presence of persistent proteinuria greater than or equal to 500 mg/dL and other recommended criteria suggested by the physician. The healthy unrelated age sex matched controls with no history of renal disease and diabetes mellitus were randomly selected and recruited from local community centers. Prior to the recruitment of subjects the ethical committee clearance was obtained from the respective medical institutions and accordingly informed consent was obtained from all the participants.

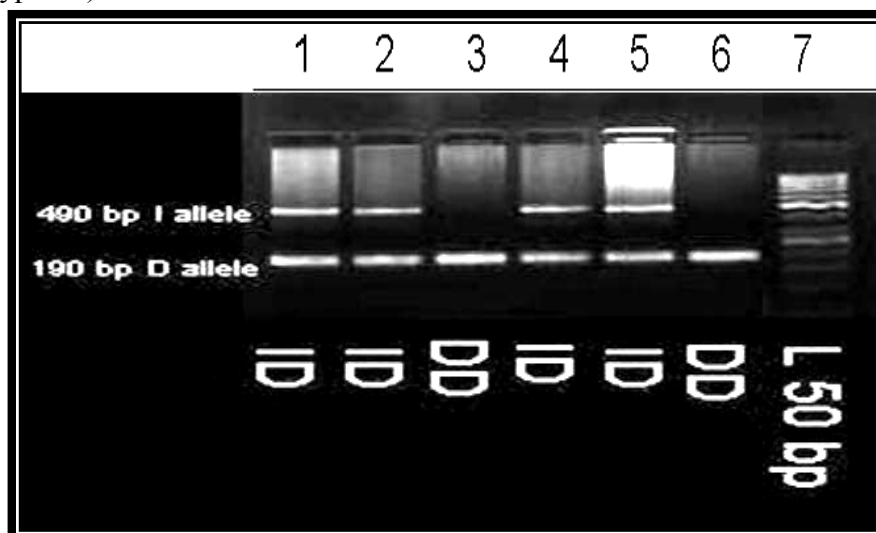
### **ACE genotyping**

Approximately 5 ml of venous blood was drawn from each of the subjects in EDTA vials and DNA was extracted from whole fresh blood using standard salting out method (Miller *et al.*, 1988) and quantification was carried out following standard spectrophotometric analysis. To detect ACE I/D polymorphism, PCR amplification was carried out in a DNA thermo cycler (Gene Amp PCR 9700 - Applied Biosystems, USA) using 20 pmoles of each of the following primer (flanking primer pair): oligonucleotide sense primer: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and anti-sense primer : 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3' (Rigat *et al.*, 1992) in a final volume of 10 $\mu$ l containing 50ng of genomic DNA, 10X PCR buffer, 25 mM MgCl<sub>2</sub>, 100 mM of each dNTP and 1 U/  $\mu$ l of Taq polymerase.

PCR was performed with a gradient standardized PCR condition with an initial denaturing time at 95°C for 6 min. Then the DNA was amplified for 35 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1:30 min and extension at 72°C for 2 min. PCR products were directly visualized in UV light using ethidium bromide staining after electrophoresis in a 2.5% agarose gel.

The amplified product was a 190 bp fragment in the presence of the deletion (D) allele and a 490 bp fragment in the presence of the insertion (I) allele. Therefore, there were three genotypes after electrophoresis:

A 490 bp band (genotype II), a 190 bp band (genotype DD), or both 490 and 190 bp band (genotype ID).



**Figure 1:** Agarose gel stained with ethidium bromide showing the amplified PCR products for ACE I/D polymorphism (Lane 7 represents the 50 bp ladder and lane No. 3, 6: DD genotype; lane: 1, 2, 4, and 5: ID genotype).

### Statistical analysis

Genotype and allele frequencies of ACE gene polymorphism were compared between type 2 diabetic patients (DM), type 2 diabetic with nephropathy patients (DN) and healthy match controls using  $\chi^2$ -test. The same test was utilised to examine if the observed genotype frequencies were in Hardy-Weinberg equilibrium among the study groups. Statistical significance was assumed at the  $p < 0.05$  level. For comparing the allelic distributions between two study groups the odds ratio (OR) with 95% confidence interval (CI) were also calculated.

### RESULTS

The average age ( $\pm$ SD) of the selected subjects having type-2 diabetes, type-2 diabetes with nephropathy and normal controls were found to be  $52 \pm 3.3$ ,  $55 \pm 4.9$  and  $50 \pm 7.2$  and the age distribution did not go beyond significant level. Sex distribution was also found to be similar in all the groups ( $\chi^2 = 1.9$ ,  $p > 0.05$ ). The number of ID, DD and II genotypes and their frequencies among the three study groups were given in Table 1. It was found that the ACE genotype distribution in DN, DM patients and in control groups were in Hardy Weinberg Equilibrium ( $\chi^2 = 0.96$ ,  $df = 2$ ,  $p > 0.05$ ;  $\chi^2 = 1.01$ ,  $df = 2$ ,  $p > 0.05$ ;  $\chi^2 = 3.58$ ,  $df = 2$ ,  $p > 0.05$ ). The table also showed that the frequency distribution of DD genotype was similar in both Type 2 diabetic as well as diabetic nephropathy patients (26.67%); whereas its frequency was found to be only 9.68 % among the controls. The distribution pattern of DD genotype within

the three population cohort did not vary significantly ( $\chi^2=3.57$ ,  $df=2$ ). When diabetic as well as diabetic nephropathy patients was taken as a single group (N=60) and a comparison was

Table 1: Genotype and allele distribution of ACE gene I / D polymorphism in Type 2 Diabetes with Nephropathy (T2DN), Type 2 Diabetes without Nephropathy (T2D) and Control Subjects.

ACE gene	DN ( n= 30)		DM ( n= 30)		CONTROL (n=31)		$\chi^2$	<i>p</i>
Genotype	No. of Cases	%	No. of Cases	%	No. of Cases	%	( <i>df</i> =2)	
DD	8	26.67	8	26.67	3	9.68	3.57	<i>p</i> > 0.05
ID	16	53.33	15	50.00	20	64.52	1.44	<i>p</i> > 0.05
II	6	20.00	7	23.33	8	25.80	0.29	<i>p</i> > 0.05
$\chi^2 = 3.681$ , <i>df</i> = 4, <i>p</i> = 0.450								
Total D phenotype	24	80.00	23	76.67	23	74.20		
II vs D phenotype( DD+ID) $\chi^2 = 0.291$ , <i>df</i> = 2, <i>p</i> = 0.8645								
Allele	T2DN ( n= 30)		T2D ( n= 30)		CONTROL (n=31)		$\chi^2$	<i>p</i>
D	32	53.33	31	51.67	26	42.00	1.86	<i>p</i> > 0.05
I	28	46.67	29	48.33	36	58.00	1.86	<i>p</i> > 0.05
$\chi^2 = 1.859$ , <i>df</i> = 2, <i>p</i> = 0.3947								

made with the control group with respect to the presence of DD genotype the same result came out ( $\chi^2=3.57$ ,  $df=1$ ,  $p>0.05$ ). The frequency of ID genotype was found to be slightly higher in healthy controls (64.52%) than the DM and DN patients (53.33% and 50.00% respectively). The same result was also found in case of II genotype where a slightly higher frequency was found among the normal controls (25.80%) than the DM (23.33%) and DN (20.00%) patients. No significant difference of frequency distribution was found between the three cohort groups with respect to ID and II genotypes ( $\chi^2=1.44$  and  $\chi^2=0.29$ ,  $df=1$ ,  $p>0.05$ ). The allele frequency of D was little higher among the DN patients (0.533) than the DM patients (0.516) and the normal controls (0.420). The frequency of I allele was also found to be little higher among the normal controls (0.580) compared to the DN patients (0.466) and DM patients (0.483). Overall it can be said that within the different study groups the distribution of ACE genotypes as well as their allele frequency did not differ significantly.

The difference in ACE genotypes as well as their allele frequency between any two different study groups along with their odds ratio was presented in Table 2. The table suggested that the frequency of DD genotype was little higher in the DM and DN patients when comparison had been made separately with the normal controls (odds ratio=3.394). But no significant difference was found between any of the two study groups ( $\chi^2=2.98$ ,  $df=1$ ,  $p=0.08$ ). The other genotypic combinations as well as allele frequency also showed insignificant difference between any of the two cohort population groups.

Table 2: ACE genotypes, allele frequency distribution and their odds ratios between different study groups

ACE Genotype	DN vs. Controls				DN vs. DM				DM vs. Controls			
	$\chi^2$ (df=1)	p	Odds ratio	95% confidence interval	$\chi^2$ (df=1)	p	Odds ratio	95% confidence interval	$\chi^2$ (df=1)	p	Odds ratio	95% confidence interval
II	0.29	0.58	0.719	0.215 - 2.393	0.09	0.75	0.821	0.239-2.814	0.05	0.82	0.875	0.272 - 2.812
ID	0.78	0.37	0.629	0.225 - 1.756	0.06	0.79	1.143	0.415-3.148	1.31	0.25	0.550	0.197 - 1.535
DD	2.98	0.08	3.394	0.804-14.319	0.00	1.00	1.000	0.318-3.141	2.98	0.08	3.394	0.804- 14.319
Allele												
I	1.59	0.21	0.632	0.309-1.292	0.03	0.86	0.935	0.457-1.915	1.16	0.28	0.676	0.331-1.381
D			1.582	0.774-3.236			1.069	0.522-2.189			1.480	0.724 - 3.025

## DISCUSSION

Diabetic nephropathy is a complex pathophysiological process which accounts for reduced life expectancy in various countries around the world and it involves the contribution of several etiologies both genetic as well as environmental in nature. So far studies on familial clustering suggested that genetic susceptibility played a major role in development of diabetic nephropathy (Krolewski *et al.*, 2001). The ACE gene was proposed to be one of the first candidate genes for developing diabetic nephropathy and there were several reasons described for this outcome (Uddin *et al.*, 2007). However there were much conflicting results for such endeavour. Ng *et al.* (2005) had undertaken a review encompassing 47 relevant studies that were published between 1994 and 2004 and supported an overall association between the ACE I/D polymorphism and diabetic nephropathy. They further noticed that this genotype-phenotype association was more marked among the Asians than the Caucasians.

In our study we presented the distribution of ACE genotypes as well as their allele frequency among the Bengali population of Eastern India and also its association with diabetic nephropathy. The study population as a whole showed a high frequency of ID genotype (56.04%) than DD (20.88%) and II (23.03%) genotype.

Studies conducted in India as well as outside India showed significant relationship between the presence of DD genotype and an increased risk of nephropathy among Type2

diabetic patients (Oh *et al.*, 1996; Yoshida *et al.*, 1996; Bhabani *et al.*, 2005; Nikzamir *et al.*, 2006; Naresh *et al.*, 2009; Haque *et al.*, 2011). As a result ACE inhibitors were widely used in the clinical management of diabetic nephropathy in several places (Ng *et al.*, 2005). Recent researches have targeted to see how the effect of those ACE inhibitors on diabetic nephropathy can be modulated by genetic variation at ACE locus (Jacobsen *et al.*, 1998).

Though in the present study a slightly high frequency of DD genotype was found among the DN and DM patients as compared to the controls the distribution did not differ beyond significant level. Thus the present study did not find any association of DD genotype with diabetic nephropathy among the Bengali population of Eastern India. Similar findings were also reported from North Indian populations (Kumar *et al.*, 2005); Poland (Grzeszczak *et al.*, 1998) and Germany (Schmidt *et al.*, 1995). In this backdrop we can say that ACE polymorphism was not a major risk factor for the development of diabetic nephropathy among the Bengali population. Observing the high incidence of diabetic as well as diabetic nephropathy among the Bengali population of Eastern India it can be presumed that there were other genetic as well as environmental factors responsible for the genetic etiology of diabetic nephropathy that might be suppressing the effect of ACE genotypes. The discovery of such unknown genes can lead to novel avenues for the prevention and treatment of this complication. There were other studies worldwide (Tamaki *et al.*, 2002; Ergen *et al.*, 2004) which support the present contention.

Table 3: The genotypic distribution and allele frequencies of the ACE I/D polymorphism in different T2DN patients in different populations of India.

Population	Genotype Distribution			Allele Frequency		Reference
	DD	ID	II	D	I	
Eastern India (n = 30)	8 (26.67%)	16 (53.33%)	6 (20.0 %)	0.53	0.47	Present Study
North India [a] (n = 59)	10 (17.0%)	32 (54.2 %)	17 (28.8 %)	0.44	0.56	Kumar <i>et al.</i> ,2005
North India [b] (n= 30)	4 (13.33%)	18 (60.00%)	8 (26.67%)	0.43	0.57	Haque <i>et al.</i> , 2011
South India [a] (n = 86)	24 (34.8%)	45 (65.2 %)	17 (19.8 %)	0.54	0.46	Viswyanathan <i>et al.</i> , 2001
South India [b] (n = 30)	15 (50.0 %)	11 (36.7 %)	4 (13.3 %)	0.68	0.32	Naresh <i>et al.</i> , 2009
$\chi^2 = 15.093, df = 8, p = 0.05$						
Pooled Indian Population (n = 235)	61(25.96%)	122(51.91%)	5 (22.13%)	0.52	0.48	

Our study also suggested that ACE polymorphism among the subjects having diabetic nephropathy differ with regard to the ethnic background of a population (Table 3). The study

did bear the testimony of the existence of ethnic variation in the distribution of ACE genotype frequency. At this stage we can presume that ACE gene, an *Alu* insertion deletion polymorphism, can be used as a suitable marker for studying genetic variation among different human populations because of its stable nature as well as representing a unique evolutionary event. Therefore while studying the susceptibility of ACE gene with the etiology of diabetic nephropathy ethnic background of the population should also be taken into consideration. Further studies from other parts of India will definitely add more information to this issue.

### **Conflict of interest**

The authors declare no conflict of interest for the present research outcome.

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