

## **Angiotensin Converting Enzyme Insertion/Deletion gene polymorphism and genomic sequence in Diabetic Nephropathy**

**Haque SF., Ahmad M, Khan AU\*; Gupta V, Khan AS**

**Department of Medicine, J.N. Medical College, Aligarh Muslim University, Aligarh, India**

**\*Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India**

### **Abstract**

Insertion/deletion polymorphism of the Angiotensin I converting enzyme genetically determines most of the plasma ACE activity. Modulation of ACE gene activity might have an important bearing on the rate of progression of renal disease, though its exact role in the nephropathy of Type 2 Diabetes is far from clear. This prospective, cross-sectional, observational study was designed to study correlation between Insertion/Deletion polymorphism of ACE gene in diabetic nephropathy. T2DM cases (n=30) were evaluated, regarding duration, onset and degree of albuminuria, renal insufficiency and hypertension. All patients underwent detailed clinical and biochemical evaluation. Genomic DNA intron 16 of the ACE gene was amplified by polymerase chain reaction (PCR), followed by sequencing. Analysis of variance ANOVA was applied to compare. A p value < 0.05 was considered significant. All calculations were performed using SPSS-11.0. The mean age of this study group was 45.21±2.34 yrs. PCR amplification of ACE gene fragment revealed I/I, (n =8) I/D (n = 18 and D/D (n = 4), alleles. Age wise, all three groups were matched (p=0.012), micro and macro-vascular complications were more prevalent in DD type. Majority (75%) of patients with II allele took longer to develop overt albuminuria, having lesser hypertension, renal dysfunction, and dyslipidemia than ID and DD allele (p<0.005). On the other hand, Urinary albumin excretion (UAE), SBP, DBP, TG, S.Cr. and LDL-C were significantly higher (p<0.005), in patients of DD type than II and ID groups. This finding suggests that patients with DD allele of the ACE gene are more likely to have progressive diabetic nephropathy with most of the micro and macro-vascular complications.

**Key words:** ACE Gene, ID polymorphism, Diabetic Nephropathy

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### **Introduction**

Genetic susceptibility to the micro-vascular complication of Diabetic nephropathy, in patients with type 2 diabetes mellitus (T2DM), is not clearly understood. Genes that seem to be of importance is angiotensin 1-converting enzyme (ACE), involved in the pathogenesis of diabetic kidney disease [1]. The ACE gene is an excellent candidate for determining prognosis for cardiovascular and renal risks in patients with Diabetes Mellitus. Interest in gene polymorphism research has led to DNA genotyping of diabetic patients with retinal, renal and cardiovascular complications. The angiotensin I converting enzyme is encoded by the human ACE gene which has been cloned and sequenced. The ACE gene is located on chromosome

17p23 and spans approximately 21kb DNA [2, 3]. The ACE gene polymorphism, first described by Rigat B, Hubert C, Corrol P and Sonbrier F [4], conventionally refers to the insertion (I) or deletion (D) of a 287 bp sequence in intron 16 of the gene. The development of a single-step method for detection of the ACE gene (I/D) polymorphism by use of polymerase chain reaction (PCR) [5] which amplifies the DNA, facilitated the large-scale research in the field of genomics of diabetes mellitus related complications. Insertion/deletion (I/D) polymorphism of the Angiotensin I converting enzyme determines most of the plasma ACE activity genetically and it has also been shown to modify response to ACE inhibition. Subjects with DD genotypes have the highest level of plasma ACE, while those with II phenotypes have lowest

levels and those with ID phenotypes exhibiting intermediate levels of plasma ACE [6] It has been reported that response of drugs was less in patients with ACE D/D genotype than in patients with I/I allele of the ACE gene. It seems likely that the risk for diabetes-associated kidney disease is magnified by inheriting risk alleles at several susceptibility loci. Genome-wide linkage studies have recently identified several chromosomal regions that likely contain diabetic nephropathy susceptibility genes. [7] Pharmacological inhibition of ACE protects against nephropathy, and retards progression to end-stage renal failure (ESRF) [8] mainly in type 1 diabetes, while it reduces cardiovascular risk, mainly in type 2 diabetes [9]. Relevance of the present study: thus evaluating the ACE I/D polymorphism is a reliable tool to identify patients at risk and those who may benefit the most of Renoprotective therapy with ACE inhibitors or ARB's. This may guide pharmacologic therapy in individual patients and help design clinical trials in progressive nephropathies. [10] Moreover, it might help optimize prevention and intervention strategies at population levels, in particular, in developing countries including India, where resources are extremely limited and 1 million patients continue to die every year of cardiovascular or renal disease.

## Materials and Methods

### Study design

Prospective, cross-sectional, observational study

### Patient selection

Type 2 diabetic patients with albuminuria attending the Medicine outpatient and Renal Clinic, of our hospital between June 2006-May 2007. All patients had established Diabetic Nephropathy—defined as persistent albuminuria ( $>300$  mg/24 h or  $>200$   $\mu$ g/min or  $>200$  mg/L) in two of three consecutive measurements on sterile urine samples—with or without renal failure (serum creatinine  $>150$   $\mu$ mol/L). Excluded from the study were patients having Reno-vascular and/or uncontrolled Hypertension, congestive heart failure, chronic kidney disease, urinary tract infection, hematuria, acute febrile illness, and patients taking ACE Inhibitors or ARB's in last one month. All patients underwent detailed clinical and biochemical evaluation: duration of Diabetes, onset of microalbuminuria, overt proteinuria, renal insufficiency and hypertension. Blood urea, serum creatinine, blood sugar fasting and post prandial, fasting lipid profile, 24-hr Urinary albumin excretion (enzyme immunoassay) were measured in all patients. Serum creatinine concentration was assessed by a kinetic Jaffe method. Lipid profile was measured by a conventional laboratory technique. The patients had diabetic diet (45 to 55% carbohydrates, 30 to 35% fat, and 15 to 20% protein) without restriction in sodium or protein intake.

### ACE genotyping

All patients gave written informed consent to study only the polymorphism of the ACE gene, thus excluding exploratory studies of other candidate genes. Oligos were designed amplify insertion/deletion of Alu repetitive element in human ACE gene. Lymphocytes were isolated from blood, and DNA was prepared by standard techniques. Polymerase chain reaction (PCR) was used to detect the two alleles of the ACE-ID polymorphism. DNA was amplified using primers and PCR-cycling conditions followed by sequencing (FIG). The study protocol was approved by the local ethical committee.

### Statistical analysis

Data are expressed as the means  $\pm$  1SD. Values for AER, not normally distributed, were logarithmically transformed before analysis and are expressed as the median and range. Differences among mean values have been evaluated by analysis of variance (ANOVA) or ANCOVA, as appropriate. A p value (two sided) of  $< 0.05$  was considered to be significant. All calculations were performed using SPSS-11.0 (Chicago, IL, USA).

## Results

The mean age of this study group was  $45.21 \pm 2.34$  yrs. The mean age of thirteen females was  $44.36 \pm 4.21$  yrs, and that of seventeen males was  $46.08 \pm 3.74$  years.

Mean systolic blood pressure was  $170.0 \pm 8.0$  and  $92.0 \pm 6.0$  mm of Hg diastolic blood pressure. Mean FBG, PPG, total cholesterol, triglyceride level were  $148.0 \pm 8.6$  mg/dl,  $242.0 \pm 9.8$  mg/dl,  $219.09 \pm 47.07$  mg/dl and  $222.06 \pm 59.58$  mg/dl respectively. Mean blood urea and serum creatinine (n=30) was  $94.0 \pm 12.8$  mg/dl and  $2.5 \pm 0.3$  mg/dl respectively. In general, those patients who had no nephropathy were older, had diabetes for longer period  $>5$  yrs, and their time to retinopathy onset was longer than the others.

**ACE Genotyping:** PCR amplification of ACE gene fragment followed by sequence analysis revealed Insertion/Deletion polymorphism. Subjects were classified according to the presence (I) or absence (D) of a 287 base pair insertion in intron 16 of the ACE gene into II, ID or DD genotypes. Two sequences have already been submitted to gene bank vide accession number: [GQ-449380](#) and [GQ-449383](#)

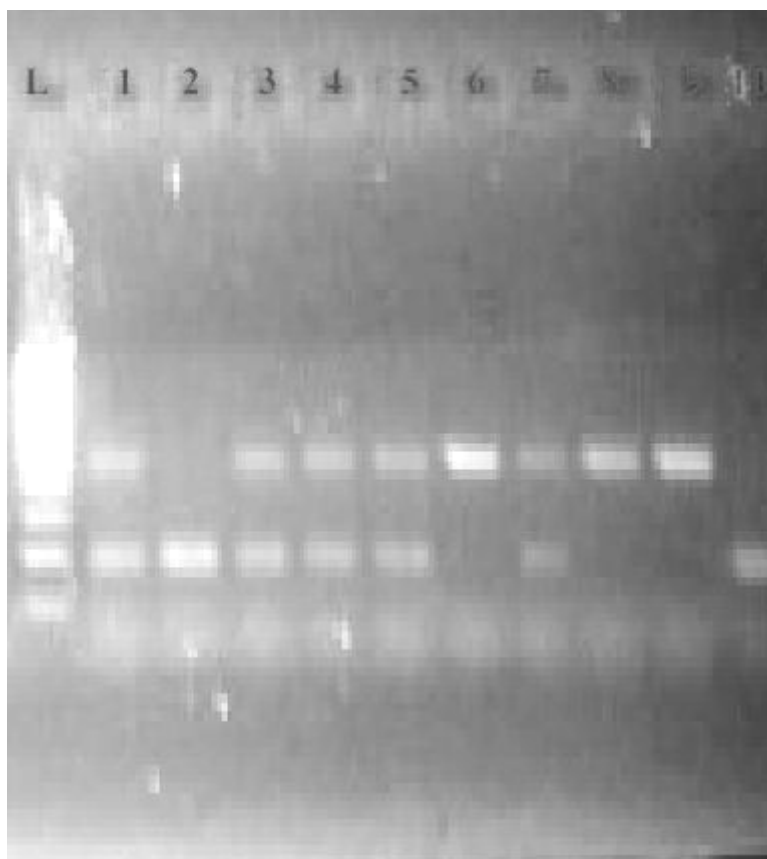
PCR primers: Forward sequence:

5'CTGGAGACCACTCCCAT CCTTTCT-3', Reverse sequence: 5'-GATGTGGCCATCACATTTCGCA GAT-3'

Patient characteristics (n =30) according to ACE I/D genotypes are shown in Table. The distribution of patients as per genotyping was ID (n = 18), II (n = 8) and DD (n = 4). The genotype frequencies for ACE I/D polymorphism were in Hardy-Weinberg equilibrium.

**Table 1.** Patient Characteristics and Distribution of ACE ID genotypes

Characteristic	ID Genotype			P value 95% C.I
	DD n = 4	ID n=18	II n= 8	
Age, yr.	43.82±1.34	47.61±2.56	45.21±2.64	0.012
Sex: Male(17) /Female(13)	2/2	10/8	5/3	
T2DM <5 yr, (%)	3 (75.0)	4(22.22)	2 (25.00)	
>5 yr, (%)	1 (25.0)	14(77.77)	6 (75.00)	
Retinopathy	4 (100%)	8 (44.44%)	2(25%)	
CAD/MI	3 (75%)	3 (75%)	0	
SBP mm Hg	178.0 ± 8.0	162.0 ±8.0	146.0 ±4.0	<0.05
DBP mmHg	92.0 ± 6.0	82.0±4.0	76.0±6.0	<0.05
UAE mg/24 hr.	1364±76.0	1193±28.0	566±12.0	<0.05
S.Creat.mg/dl	3.85 ±0.68	2.87 ±0.24	1.85 ±0.34	<0.05
T.Chol.mg/dl	230.0±32.0	225.8±46.0	176.5±26.0	0.020
TG mg/dl	280.9±48.0	232.4±28.0	190.8±30.2	<0.05
LDL mg/dl	168.4±28.3	150.3±24.2	104.1±18.6	<0.05
HDL mg/dl	41.4 ±4.8	45.5±5.1	52.2±4.2	<0.2



**Figure 1.** PCR amplification of human ACE gene from 10 blood samples, the PCR fragments amplified were loaded on 2% agarose gel.

All the patients were comparatively evaluated. Age wise, all three groups were matched (p=0.012), micro and macro vascular complications were more prevalent in DD

type, where retinopathy and CAD was present in 100% and 75% cases, respectively. In patients having genotype II (n = 8), majority (75%) of patients took longer time >5

yr, to develop overt albuminuria, having lesser degree of hypertension, renal dysfunction, and dyslipidemia than ID and DD genotypes ( $p < 0.005$ ). On the other hand, Urinary albumin excretion (UAE), SBP, DBP, TG, S.Cr. and LDL-C were significantly higher in patients of DD type than II and ID groups. ( $p < 0.005$ )

## Discussion

Angiotensin-I converting enzyme (ACE) gene is one of the most intensely studied genes because of the key role it plays in the renin-angiotensin system (RAS). ACE gene is located on chromosome 17q23 and consists of 26 exons and 25 introns. The insertion deletion (I/D) polymorphism in this gene refers to an Alu repetitive sequence 287 bp long, in intron 16, found in three forms: D/D and I/I homozygotes and I/D heterozygotes. Alu insertion polymorphisms, like ACE I/D polymorphism, are also suitable markers for studying genetic variation in human populations. They can be easily detected by PCR amplification and gel electrophoresis and they are stable markers that represent a unique evolutionary event.

The distribution of the ACE genotypes differs between races and it is used as a marker in population structure analyses [11]. In our study ID genotype was the most frequent allele present in 60.0%, followed by II in 26.66% and DD found in only 13.33% of total cases of diabetic nephropathy. T Golmohamadi, A Nikzamir, M Nakhjavani, M Zahrai, A Amirzargar and R Saffari, in an Iranian cohort found the frequency of DD, ID and II genotypes in patients with nephropathy to be 30.6%, 55.3%, 14.1%, respectively [12]. While in a North Indian diabetic nephropathy population the frequency was 17.0%, 54.2%, 28.8% for DD, ID and II genotypes respectively [13].

In the present study 73.33% patients belonged to ID and DD genotype. Clinical correlation revealed that most patients in this group have macro and micro vascular complications: CAD, retinopathy, greater degree of proteinuria, severe renal insufficiency ( $p < 0.05$ ). We conclude from the study that haplotypes including the deletion allele (D) of the ACE gene are associated with greater risk of diabetic nephropathy compared with haplotype insertion (I) allele. Further the prevalence, onset and progression of diabetic nephropathy was greater in patients with DD genotype. Jeffers et al. have also shown an association between ACE DD genotype and Diabetic Nephropathy [14].

The association of DD allele with Diabetic nephropathy differ from 13.33% (present study). to 22.75% in South India [15] 28.8% in Brazil [16] and 30.6% in Iran [12]. This difference is attributed mainly to difference in frequency of ACE genotypes in patients with diabetic nephropathy, diabetic without nephropathy and non-diabetics in different ethnic groups [18]. The DD genotype is known as an independent risk factor has a high prognostic value

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for onset and progression of diabetic nephropathy in T2DM. Further, many studies have established that the DD genotype leads to higher ACE expression and activity and may be predispose individuals to T2DM and its complications. All previous studies in non-diabetic and diabetic nephropathies have demonstrated that the deletion polymorphism of the ACE gene, particularly the homozygote DD, is a risk factor for an accelerated loss of kidney function. Besides Diabetes mellitus, it was found that ACE insertion/deletion polymorphism, is associated with essential hypertension [19-20]. Further individuals who are homozygous for the D allele of the ACE gene are more likely to have essential hypertension. [21].

Polymorphisms in the RAS system are associated with clinically significant renal, cardiovascular disease morbidity and altered serum ACE activity and thought to occur through a pro-inflammatory mechanism. Individuals with DD genotype have twice as much as tissue and plasma ACE concentrations as I/I subjects, with ID subjects having intermediate levels. The explanations for the deleterious effect of the deletion polymorphism on albuminuria may be elevated intra-renal angiotensin II formation and/or insufficient angiotensin converting enzyme in DD type individuals [22]. A meta-analysis showed that the risk of nephropathy was increased in the presence of DD or ID genotypes in Asian patients with types 2 diabetes [23].

## Conclusion

Consequently, genotypic abnormalities in the renin-angiotensin system have emerged as potential risk factors for the development of diabetic nephropathy. And evaluating the ACE I/D polymorphism is a reliable tool to identify patients at risk of Diabetic nephropathy and those who may benefit the most from ACE inhibitors or ARB's in retarding the progression of Chronic Kidney Disease and ESRD. This may help optimize prevention and intervention strategies at population levels, especially in developing countries including India, where renal replacement therapies are neither accessible nor affordable to millions of diabetics who continue to die every year of cardiovascular or renal disease

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**Correspondence to:**

S .F. Haque  
Department of Medicine,  
J.N. Medical College Hospital  
Aligarh Muslim University  
Aligarh 202002, India

