

## ANGIOTENSIN CONVERTING ENZYME (ACE) GENE INSERTION DELETION POLYMORPHISM AND ITS ASSOCIATION WITH TYPE 2 DIABETIC NEPHROPATHY

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### ABSTRACT

**Background :** Long term type 2 diabetes is a major and important cause of nephropathy. ACE responsible for glomerular pressure change and release of glomerulosclerosis mediators. In ACE gene there exists a polymorphism within intron 16 in the form of either presence (I) or absence (D) of a 287 base-pair fragment resulting in 3 possible genotypes-II, ID, DD. This polymorphism is associated with variability in circulating levels of ACE. II genotype having lowest ACE levels, DD genotype having highest and ID genotype having intermediate levels. **Aims & Objectives:** 1. To find correlation of DD polymorphism with diabetic nephropathy. 2.To assess serum ACE activity in relation to this polymorphism. **Materials & Methods:** Genotype analysis done in 30 healthy controls, 30 diabetics with nephropathy and 30 diabetics without nephropathy by polymerase chain reaction. Phenotype analysis done by measuring serum ACE activity. **Results:** Diabetic nephropathy patients had higher frequency of DD genotype compared to controls and diabetics without nephropathy (56.7% compared to 6.7% and 20.0% P=0.000) and Odds ratio of 2.7 among diabetic nephropathy patients and controls. ACE activity elevated in DD genotype ( P = 0.000) **Conclusion:** ACE DD genotype is a risk factor for Diabetic nephropathy and is associated with increased serum ACE activity.

**Key words:** Type 2 Diabetes, Diabetic Nephropathy, ACE gene, DD genotype, ACE activity.

### INTRODUCTION

Diabetic nephropathy is a more common cause of chronic kidney disease especially in developing countries. Genetic susceptibility to the micro-vascular complications of Diabetic nephropathy, in patients with Type 2 Diabetes mellitus (T2DM), is not clearly understood. Genome-wide linkage studies have recently identified several chromosomal regions that likely contain diabetic nephropathy susceptibility genes<sup>1</sup>. Studies have shown that rennin –angiotensin system may play an important role in the development of nephropathy in Type 2 Diabetes

mellitus. Genes that seem to be of importance is angiotensin converting enzyme (ACE), involved in the pathogenesis of diabetic kidney disease<sup>2</sup>. The ACE gene is an excellent candidate for determining prognosis for cardiovascular and renal risks in patients with Diabetes Mellitus. ACE polymorphism may be a potential predictor for development of nephropathy in Type 2 DM<sup>3,4</sup>.

The development of a single-step method for detection of the ACE gene (I/D) polymorphism by use of polymerase chain reaction (PCR)<sup>5</sup> which amplifies the DNA, facilitated the large-scale research in the field of genomics of diabetes

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mellitus related complications.

ACE catalyses production of the vasoactive peptide Angiotensin II from its precursor Angiotensin I. Within the diabetic kidney the effects of Angiotensin II include an increase of intraglomerular pressure and glomerular filtration rate. In addition to its hemodynamic effects Angiotensin II stimulates the production or release of several cytokine mediators of glomerulosclerosis such as Osteopontin, Platelet derived growth factor, Fibronectin and Transforming growth factor  $\beta^3$ .

The ACE gene located on chromosome 17q23<sup>6,7</sup> contains a polymorphism in intron 16<sup>8,9,10</sup> of an Insertion / Deletion (I/D) of a 287 –bp repeat sequence. Insertion allele produces 490 bp product and deletion allele produces 190bp product which results in 3 possible genotypes of ACE gene as II, DD, ID.

Insertion/deletion (I/D) polymorphism of the Angiotensin I converting enzyme determines most of the plasma ACE activity genetically. Subjects with DD genotypes have the highest level of plasma ACE, while those with II genotypes have lowest levels and those with ID genotypes exhibiting intermediate levels of plasma ACE<sup>11</sup>. 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.

The primary objective of the study was to find out the distribution of ACE gene polymorphism in healthy controls, in Type 2 diabetic patients without nephropathy, in type 2 diabetic patients with

nephropathy and to study the relationship between DD polymorphism and diabetes with nephropathy.

## **MATERIALS AND METHODS :**

### **Study population:**

#### **Cases:**

The study sample comprised of 30 type 2 diabetics with nephropathy (14 males, 16 females), and 30 type 2 diabetics without nephropathy (11 males, 19 females) of mean age 53.9 years . Patients attending diabetic OPD were selected and categorised as with nephropathy and without nephropathy based on early morning urine albumin creatinine ratio. Patients who were known hypertensives and with blood pressure above 140/90mmHg, heart disease patients and patients on ACE inhibitors were excluded from the study.

#### **Controls:**

30 age matched healthy individuals (15 males and 15 females) with normal albumin creatinine ratio were selected from patients attending master health check up.

#### **Methods:**

Fasting plasma glucose by GOD-POD method, Blood urea by GLDH method, Serum and Urine Creatinine by modified Jaffes, Urine microalbumin by immunoturbidimetry.

#### **Urine microalbumin :**

##### **Principle:**

Latex particles coated with specific antibodies anti-human albumin are agglutinated when mixed with samples containing  $\mu$ ALB. The agglutination causes an absorbance change, dependent upon the  $\mu$ ALB contents of the sample

that can be quantified by comparison from a calibrator of known  $\mu$ ALB concentration. Wavelength used: 540 nm.

### ACE GENE POLYMORPHISM SCREENING:

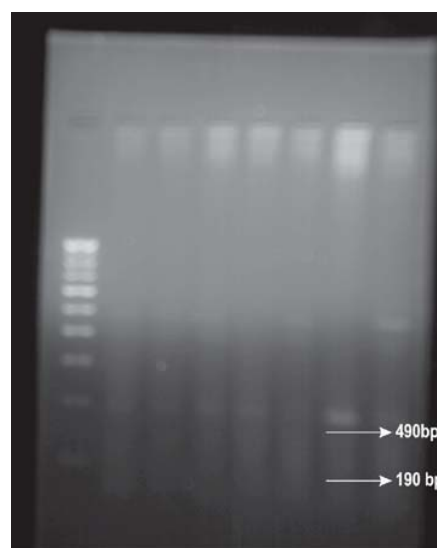
DNA was extracted from Buffy coat by Genei-whole blood DNA extraction kit and Helini biolabs- mammalian whole blood spin column extraction kit method and using ACE gene forward primer 5' - CTGGAGACCACTCCCATCCTTTCT-3' and reverse primer 5' - GATGTGGCCATCAATTCGTCAGAT - 3' genomic DNA (1 $\mu$ g) was amplified in 12.5 $\mu$ l reaction mixture containing 0.3 $\mu$ mol/L of each primer and red dye master mix (Bangalore Genei) containing 100 $\mu$ mol/L of each dNTP, 2.5 $\mu$ L of 10x reaction buffer and 0.6 unit of Taq DNA polymerase. After the DNA was denatured for 5 minutes at 94°C, the reaction mixture was subjected to 30 cycles of denaturation for one minute at 94°C, 1 minute of annealing at 58°C and 1 minute of extension at 72°C. Final extension was carried over at 72°C for 10 minutes.

Amplification products were separated by electrophoresis on a 2% agarose gel, and visualized under ultraviolet light after ethidium bromide staining. The PCR product is a 190 bp fragment in the presence of a deletion (D) allele, and a 490 bp fragment in the absence of a deletion (I) allele. Thus each DNA sample revealed one of three possible patterns after electrophoresis: a 490 bp band (II genotype), a 190 bp band (DD genotype), or both 490 and 190 bp bands (I/D genotype) (Rigatet al., 1992)<sup>12</sup>. Analysis was done using a low molecular weight DNA ladder - Bangalore genei.

Fig 1. Extracted DNA (lane 2 to 8) was tested on 1% agarose gel using 1kb ladder (lane 1) Ladder shows 10000, 8000, 7000,6000, 5000, 4000, 3000, 2000,1000 bp fragments



Fig 2: Agarose gel electrophoresis of PCR products. Deleted allele has 190bp product, inserted allele has 490 bp product. Lane 1 shows ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000bp), lanes 2,3,4,8 shows both the products and indicating ID genotype, lanes 5,7 shows only 190bp product indicating DD genotype, lane 6 shows only 490 bp product indicating II genotype.



**PHENOTYPE ANALYSIS:**

Serum ACE activity was measured using Trinity Biotech kit at 340 nm in UV spectrophotometer (Turbidimetry).

**Principle:**

The following reaction is catalysed by ACE:  
 $FAPGG \rightarrow FAP + Glycylglycine$   
 FAPGG (N-[3-(2-furyl)acryloyl]-L-phenylalanyl glycylglycine) is hydrolysed to furylacryloylphenylalanine (FAP) and glycylglycine. Hydrolysis of FAPGG results in a decrease in absorbance at 340 nm. The ACE activity in the sample is determined by comparing the sample reaction rate to that obtained with the ACE calibrator.

**Procedure:**

The temperature of the reaction mixture was maintained at 37°C. To 1 ml of ACE reagent 100µL of calibrator or serum sample added, mixed well. Constant temperature maintained. And after 5 min absorbance recorded, which is the initial absorbance. Exactly after 5 min again absorbance recorded. This is the final absorbance.

**Calculation:**

$$\Delta A \text{ per } 5 \text{ min (Test)} = \text{Initial A Test} - \text{Final A Test}$$

$$\Delta A \text{ per } 5 \text{ min (Calibrator)} = \text{Initial A Calibrator} - \text{Final A Calibrator}$$

$$ACE \text{ (U/L)} = \frac{\Delta A / 5 \text{ min of Test}}{\Delta A / 5 \text{ min of Calibrator}} \times \text{Activity of calibrator}$$

Absorbance values were calculated using an ACE Calibrator with an activity of 50µL at 37° C.

**RESULTS AND STATISTICAL ANALYSIS**

1. BMI, Waist Hip ratio, Fasting plasma glucose, Blood urea, Serum Creatinine, urine creatinine and urine microalbumin were compared between groups by Anova and chi-square test.
2. Genotype frequency distribution between cases and controls were compared with a  $\chi^2$  test and odds ratio calculated.
3. ACE activity correlated with genotype by chi-square test
4. Logistic regression analysis was performed to evaluate the interaction between ACE genotypes II/DD/ID and other variables in relation to the prevalence of diabetic nephropathy.

**TABLE-1 Comparison of parameters between and within groups.**

Parameters	Groups	df	Mean Square	F	Sig.
Waist circumference(cm)	Between Groups	2	36.303	.469	.627
	Within Groups	87	77.474		
	Total	89			
Hip circumference (cm)	Between Groups	2	206.875	2.616	.079
	Within Groups	87	79.071		
	Total	89			
Waist Hip ratio	Between Groups	2	.018	6.495	.002
	Within Groups	87	.003		
	Total	89			
Fasting Plasma Glucose (mg/dL)	Between Groups	2	61941.878	18.351	.000
	Within Groups	87	3375.326		
	Total	89			
Blood Urea (mg/dL)	Between Groups	2	16.411	.363	.697
	Within Groups	87	45.245		
	Total	89			
Serum Creatinine (mg/dL)	Between Groups	2	.044	3.570	.032
	Within Groups	87	.012		
	Total	89			
Urine creatinine (g/L)	Between Groups	2	.095	.968	.384
	Within Groups	87	.099		
	Total	89			
Urine MicroAlbumin (mg/L)	Between Groups	2	39872.934	109.299	.000
	Within Groups	87	364.806		
	Total	89			

Table 1 shows various parameters compared between and within groups. Waist hip ratio, Fasting plasma glucose and Urine microalbumin values show significant P values.

**TABLE-2 : Albumin creatinine ratio**

Groups	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	82852.970	2	41426.485	107.978	.000
Within Groups	33378.026	87	383.655		
Total	116230.996	89			

Table 2 shows significant P value for Albumin Creatinine ratio between groups. Albumin creatinine ratio of  $\leq 23$  mg/g of creatinine for males and  $> 32$  mg/g of creatinine for females is considered as nephropathy<sup>13</sup>

**TABLE-3 : ACE gene polymorphism**

Genotype	Controls 30	DM without Nephropathy 30	DM with Nephropathy 30	P value
DD	2(6.7%)	17(56.7%)	6(20.0%)	0.000
ID	16(53.3%)	9(30.0%)	13(43.3%)	
II	12(40.0%)	4(13.3%)	11(36.7%)	

Table 3 shows that Genotype distribution and Allele frequencies of human ACE gene in patients with DM with nephropathy, DM without nephropathy and control subjects. ACE genotype distribution was in agreement with the Hardy-Weinberg expectations. DD genotype was more frequent among nephropathy cases (56.7 %) when compared to controls (6.7%). In contrast ID and II was more common among controls (53.3% & 40.0%) .P value is 0.000. ID genotype is more common among controls when compared to cases.

**TABLE-4: ANOVA ACE ACTIVITY**

Groups	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11638.156	2	5819.078	13.810	.000
Within Groups	36660.167	87	421.381		
Total	48298.322	89			

Table 4 shows the ACE activity between groups and within groups. Table 5 shows the multiple logistic regression analysis of variables.

It was observed that ACE activity was elevated in DD genotype. P value of 0.000 observed in between nephropathy and control and without nephropathy groups for ACE activity.

**TABLE 5: MULTIPLE LOGISTIC REGRESSION ANALYSIS**

Parameters	B	Std. Error	Wald	df	Sig.	Exp(B)
Intercept	31.053	73.548	.178	1	.673	
Age	-.578	1.707	.115	1	.735	.561
Sex	-30.776	77.773	.157	1	.692	4.307E-14
BMI	2.222	5.635	.156	1	.693	9.226
Waist Hip Ratio	390.232	1012.594	.149	1	.700	2.988192825969476+169
Fasting Plasma Glucose	-1.289	3.185	.164	1	.686	.276
Blood Urea	1.383	3.753	.136	1	.712	3.988
Serum Creatinine	-181.882	438.270	.172	1	.678	1.023E-79
Urine Creatinine	35.281	94.127	.140	1	.708	2101600481290694.000
Urine Microalbumin	-4.077	11.117	.134	1	.714	.017
ACR	1.243	3.730	.111	1	.739	3.465
ACEPolymorphism	+2.7371	66.382	.170	1	.680	1.297E-12
ACEActivity	-3.74	.755	.246	1	.620	.688

**DISCUSSION**

Renal failure is an outcome of complex pathophysiological process resulting from multiple etiologies with contribution from both genetic and environmental factors. The factors that initiate ESRD in patients remain unknown, although diabetes mellitus and hypertension are known important risk factors responsible for the occurrence of this irreversible disease. Consequently, a marked ethnic difference in the risk of developing ESRD exists and believed that genetic factors are likely to be responsible for such differences (Freedman et al. 1997).

The French and Belgian GENEDIAB study demonstrated that the D allele was associated with both an increased incidence and severity of diabetic nephropathy in a large group of type 1 diabetic patients<sup>17</sup>. In the diabetic nephropathy group, there was an elevated association with the D allele. This was in keeping with the observations in several populations. Follow-up studies of the patients have to be carried out to study the relationship of the genotypes with the severity and the rate of decline of kidney function.

The primary objective of the study was to find the pattern of distribution of ACE gene polymorphism in healthy controls, in type 2 diabetics without nephropathy and type 2 diabetics with nephropathy and to study the relationship between DD gene polymorphism and diabetic nephropathy.

Although data from caucasians failed to confirm an increased risk for development of nephropathy associated with D allele, a role of this genetic marker in south Asians with NIDDM cannot be ruled out.

This study demonstrated a positive association between the D allele (ID and DD genotype) of the ACE polymorphism and diabetic nephropathy in type 2 diabetic patients. Several Japanese studies had also found the D allele to be an independent risk factor for diabetic nephropathy in type 2 diabetic patients<sup>14,15,16</sup>. The odds ratio noted in our study for the association of D+ allele with nephropathy (OR=3.00).

#### Future Prospects :

1. ACE genotype screening in families with type 2 diabetes.
2. To monitor progression / severity of nephropathy.

#### Limitations of the study:

1. Comparison of only a small sample of controls and Diabetic nephropathy cases in our study may not be enough to say that DD polymorphism is risky.
2. Also we have not confirmed the DD genotypes using insertion specific primers as previous studies showed that there is possibility of mistyping<sup>17</sup> due to preferential amplification of D allele and all the DD genotype positive cases to be confirmed by insertion specific primer.

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Received on 04/01/2016, Modified on 06/02/2016, Accepted on 08/03/2016