Association of Angiotensin-Converting Enzyme Gene Polymorphisms and Nephropathy in Diabetic Patients at a Tertiary Care Centre in South India

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ABSTRACT

BACKGROUND: Genetic polymorphisms of the angiotensin-renin pathway have been thought to influence the development of diabetic nephropathy. However, there are conflicting results regarding this association in previous studies on populations with varying ethnicity.

AIMS: Primary aim was to compare the frequency of distribution of angiotensin-converting enzyme (ACE) gene (insertion/deletion [I/D]) polymorphism in Tamilian Indian type 2 diabetic individuals with and without microalbuminuria. Secondary objective was to compare the frequency of distribution of the 3 genotypes in diabetic patients with urinary albumin/creatinine ratio (ACR)<30 mg/dL, urinary ACR=30 to 300 mg/dL, and urinary ACR>300 mg/dL.

METHODS: A total of 179 consecutive diabetic individuals between 40 and 70 years, from Puducherry and Tamilnadu of Dravidian descent participated in the study conducted from 2012 to 2014. Inclusion criteria were as follows: age ≥40 years and duration of type 2 diabetes mellitus for ≥5 years. Patients were divided into 2 groups based on ACR values. Group 1 consisted of 50 individuals with urinary ACR<30 mg/g of creatinine, and group 2 consisted of 129 individuals with urinary ACR>30 mg/g. Angiotensin I–converting enzyme (ACE) gene polymorphism was determined by allele-specific polymerase chain reaction method using a primer pair flanking the polymorphic region of its intron 16. Furthermore, group 2 patients were subdivided into those with urinary ACR=30 to 300 mg/g of creatinine and those with urinary ACR>300 mg/g of creatinine, and distribution of ACE gene polymorphism was compared in the three groups.

STATISTICS: Statistical analysis was done using SPSS version 17.0. Independent Student t test was used to compare mean values between the 2 groups. Odds ratio was calculated for testing association between ACE gene (I/D) polymorphism and presence of microalbuminuria. P < .05 was considered significant. Comparison of ACE genotypes among 3 groups of patients (ACR<30 mg/g, ACR=30-300 mg/g, and ACR>300 mg/g) was done using 1-way analysis of variance with Bonferroni multiple comparison test as post hoc analysis.

CONCLUSIONS: Heterozygous I/D genotype was more frequent in the study population (45.8%) than the other genotypes. There was no difference in the genotype distribution in patients with varying levels of albuminuria.

KEYWORDS: Angiotensin I–converting enzyme (ACE) gene polymorphism, polymorphism, diabetic nephropathy, South India

Background

Diabetes is a leading cause of chronic kidney disease worldwide, and it accounts for 31% to 40% of end-stage renal disease cases in India.¹,² Although long-standing diabetes and poor glycaemic control are risk factors for diabetic nephropathy, ethnic variability in individual's genotype also increases the susceptibility to these complications.³

Renin–angiotensin pathway is closely implicated in causation of diabetic nephropathy by causing effenter arteriolar constriction in the glomerulus and raising the intraglomerular pressure. The angiotensin-converting enzyme (ACE) converts angiotensin I to angiotensin II in the renin–angiotensin system (RAS). Angiotensin II is a vasoconstrictor. The ACE also metabolizes bradykinin which is a vasodilator. The RAS regulates blood pressure by these mechanisms.

The ACE gene polymorphisms include several single-nucleotide polymorphisms (SNPs), only 34 of which are located in the coding regions.⁴ The insertion/deletion (I/D) polymorphism in the 287 base pair in intron 16 of ACE gene, on the long arm of chromosome 17q23 (SNP ID: rs1799752) is associated with variation in ACE activity.⁵ This polymorphism has been studied for association with diabetic nephropathy, with conflicting results.⁴,⁷ The aim of this study was to assess the distribution of ACE gene polymorphism in Tamilian Indian type 2 diabetic individuals attending JIPMER.
(Jawaharlal Institute of Postgraduate Medical Education and Research) hospital and determine whether there is an association between I/D polymorphism and severity of nephropathy.

Material and Methods

Ethics

Institute ethics committee approval was obtained before the start of the study (JIP/IEC/1/2012/26).

Study setting

The study was performed in the Department of Medicine, in collaboration with the Department of Pharmacology in JIPMER from 2012 to 2014.

Study subjects

A total of 179 consecutive type 2 diabetic individuals, with at least 5 years duration of diabetes, attending diabetic outpatient clinic, were recruited in this study, after obtaining informed consent. They were 40 to 70 years of age, from Puducherry and Tamilnadu, of Dravidian descent and were residing in this area for at least 3 successive generations. Patients with fever, congestive cardiac failure, on haemodialysis and postrenal transplant patients were excluded. Diabetes is defined as fasting plasma glucose value more than 126 mg/dL or 2-hour postprandial value of more than 200 mg/dL or glycated haemoglobin more than 6.5%. After a detailed history and examination, spot urine sample for albumin/creatinine ratio (ACR) estimation (by immunoturbidimetric method) and 5 mL of venous blood sample were taken in EDTA-containing tubes (100 μL of 10% EDTA as anti-coagulant) for genotyping. Plasma lipid profile, fasting and postprandial blood sugar, renal function parameters, and glycated haemoglobin (HbA1c) were also estimated. Patients were divided into 2 groups based on ACR values. Nephropathy was defined as patient’s spot urine ACR value of more than 200 mg/dL or glycated haemoglobin more than 6.5%. After a detailed history and examination, spot urine sample for albumin/creatinine ratio (ACR) estimation (by immunoturbidimetric method) and 5 mL of venous blood sample were taken in EDTA-containing tubes (100 μL of 10% EDTA as anti-coagulant) for genotyping. Plasma lipid profile, fasting and postprandial blood sugar, renal function parameters, and glycated haemoglobin (HbA1c) were also estimated. Patients were divided into 2 groups based on ACR values. Nephropathy was defined as patient’s spot urine ACR value more than 30 mg/g of creatinine. Group 1 consisted of 50 individuals with urinary ACR < 30 mg/g of creatinine, whereas group 2 consisted of 129 individuals with urinary ACR > 30 mg/g.

DNA extraction

For genotyping, plasma was separated from whole blood by centrifugation, and DNA was extracted from peripheral blood leukocytes by standard phenol-chloroform method. The extracted crude DNA was suspended in Tris-EDTA buffer and stored at 4°C. The DNA samples were quantified using multi-channel spectrophotometer (TECAN Infinite M200; Tecan, Männedorf, Switzerland) at absorbance A260nm. Diluted DNA samples of 100-ng/μL concentration were stored at 4°C for further use.

PCR amplification of the target DNA fragment

All the reactions were performed according to the method as described in previous studies. Angiotensin I-converting enzyme (ACE) gene polymorphism was determined by allele-specific polymerase chain reaction (PCR) method using a primer pair flanking the polymorphic region of its intron 16. In this method, the presence or absence of 287-base pair (bp) fragment in the DNA sequence of intron 16 of ACE gene was determined through amplification thus producing an amplicon of size 490 bp (for insertion allele), 190 bp (for deletion allele), or both products (for presence of both alleles). Three primers are used for the purpose in this study as follows: forward primer for I/D 5′-CTGGAGAGGCCATCCCATCCTTTCT-3′, reverse primer for I/D 5′-GACGTGGCCATCACATTCGTCAGAT-3′, and insertion-specific primer 5′-TTTTGAGACGGAGTCTCGCTC-3′. All the 3 primer sequences were considered based on previous studies. Lyophilized primers were commercially purchased and reconstituted as stock followed by its dilution for a working concentration of 20 pmol/μL. The PCR reaction mix comprising 1.0-μL template DNA along with above primers (0.5 μL each), 0.5 μL of dNTPs mix, 0.625 μL of 25-mM magnesium chloride solution, Taq polymerase enzyme, and its standard buffer was prepared and the volume was made up to 25 μL using Milli-Q water. The PCR conditions standardized for amplification of ACE gene polymorphism in our laboratory conditions were as follows. Step 1: initial denaturation at 94°C for 5 minutes; step 2: 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1.5 minute, and extension at 72°C for 2 minutes; step 3: final extension at 72°C for 2 minutes; and step 4: final cooling at 25°C for 1 minute.

Genotyping

Angiotensin I–converting enzyme (ACE) gene polymorphism was classified as I/I, I/D, D/D. Amplified PCR products were analysed by 2% agarose gel electrophoresis. The sizes of different fragments were 490 bp (I/I), 190 bp (D/D), and 490 and 190 bp (I/D) (Figure 1). The fragments reported as homozygous deletion (D/D) were retyped using the third insertion-specific primer. This was done to rule out mistyping due to preferential amplification of the deletion fragment over the longer insertion fragment. Positive and negative controls were included in genotyping. The observed allele frequency was checked for Hardy-Weinberg equilibrium.

Statistics

Statistical analysis was done using SPSS version 17.0. The allele and genotype frequencies of study subjects were determined by direct gene counting. The observed frequencies were compared with expected frequencies and tested for Hardy-Weinberg equilibrium. Odds ratio was calculated for testing association between ACE gene (I/D) polymorphism and presence of microalbuminuria. Independent Student t test was used to compare mean values between the groups. P < .05 was considered significant. Comparison of ACE genotypes among 3 groups of patients (ACR < 30 mg/g, ACR = 30-300 mg/g, and ACR > 300 mg/g) was done using 1-way
analysis of variance (ANOVA) with Bonferroni multiple comparison test as post hoc analysis.

Results
Over a study period of 2 years, 179 type 2 diabetics participated in this study. In all, 50 had ACR less than 30 mg/g of creatinine (group 1) and 129 had ACR more than 30 mg/g of creatinine (group 2) in their spot urine sample. Clinical and laboratory characteristics are given in Table 1. All diabetic patients were genotyped for the ACE gene. I is the insertion allele and D is the deletion allele. The allele and genotype frequencies were in Hardy-Weinberg equilibrium (Table 2). The genotype frequencies were compared among group 1 and group 2 subjects and were not found to have statistically significant difference ($P > .05$) (Table 3). On combining the heterozygous and homozygous mutants for D allele and comparing with I/I genotype frequencies in the 2 groups using $\chi^2$ test, no statistical significance was found (Table 4). We also compared the genotype distribution among 3 groups of subjects, based on whether their urinary ACR was less than 30 mg/g of creatinine, 30 to 300 mg/g creatinine, or more than 300 mg/g creatinine. One-way ANOVA with Bonferroni multiple comparison test as post hoc analysis was done, and the $P$ value was .2902, considered statistically not significant. Hence, there was no difference in the genotype distribution of patients with varying levels of albuminuria (Table 5).

Discussion
Functional ACE gene polymorphisms are involved in pathogenesis of nephropathy through RAS and kinin–kallikrein system. The distribution of ACE genotype varies according to the ethnicity of the population. In this study, a homogeneous population of South Indians of Dravidian descent were included. Frequency of I allele was 62% and that of D allele was 38% in the diabetic cohort. Heterozygous I/D genotype was more frequent in the study population (45.8%) than the other genotypes. This polymorphism is also the most common among diabetic patients from Punjab, Haryana, Himachal Pradesh, Assam, and hypertensive diabetic patients from Hyderabad in South India.

We did not find any association between D allele and D/D polymorphism with occurrence of nephropathy. This is similar to previous studies from United Kingdom, Japan, Spain, Denmark, Korea, Germany, and North India. D allele was not
associated with nephropathy in the European participants. Polish diabetic patients with D/D polymorphism had higher ACE levels, but there was no difference in genotype distribution among albuminuric and nonalbuminuric participants. Chinese studies also demonstrate the lack of association between ACE gene I/D polymorphism and nephropathy. Among Punjabi population in North India, the genotype distribution and allele frequencies did not differ among type 2 diabetics on haemodialysis and controls. However, our results are in contrast to previous studies from South India which reported an association of D/D polymorphism with nephropathy in type 2 diabetes subjects. However, number of participants studied was small in these studies. Another study from Gujarat also found microalbuminuria to be more prevalent in those patients with insulin-dependent diabetes mellitus who had D/D polymorphism. Mooyart et al reported that genetic variants in the renin-angiotensin were associated with nephropathy in diabetic patients. This may be due to ethnic variability in genotype distribution and possible interaction with other SNPs in the RAS pathway. There is need to explore the interaction of I/D with eNOS (endothelial nitric oxide synthase) and MTHFR (methylene tetrahydrofolate reductase) polymorphisms. This is a cross-sectional study, and a longitudinal follow-up of study participants will provide better understanding of the genetic influence on the pathogenesis of albuminuria in non-nephropathic participants of this study. Assessing ACE activity levels in those with D/D polymorphism should also be done and correlated with extent of albuminuria in nephropathic diabetic patients. To conclude, this study contributes to the paucity of data regarding ACE gene polymorphism and its association with diabetic nephropathy in South Indian (Tamilian) population.

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**Author Contributions**

MW designed the study, defined the intellectual content and acquired the data. MW, RN and SV analyzed the data, did statistical analysis and wrote the manuscript. MW, RS, AKD and CA reviewed the literature and edited the manuscript.

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| Table 3. Comparison of ACE gene polymorphism among 2 groups of patients. |
|-------------------------|-------------------------|-------------------------|-------------------------|
| **ACE GENOTYPE**       | **ACR < 30 MG/G OF CREATININE, %** | **ACR > 30 MG/G OF CREATININE, %** | **OR (95% CI)** |
| I/I                    | 18 (36)                  | 52 (40.3)                | 1                       |
| I/D                    | 26 (52)                  | 56 (43.4)                | 0.760 (.373, 1.547)     |
| D/D                    | 6 (12)                   | 21 (16.3)                | 1.235 (.430, 3.546)     |
| **Total**              | 50                       | 129                      |                          |

Abbreviations: ACE, angiotensin-converting enzyme; ACR, albumin/creatinine ratio; CI, confidence interval; OR, odds ratio.

| Table 4. Combination of heterozygous and homozygous mutants. |
|-------------------------|-------------------------|-------------------------|
| **ACE GENOTYPE**       | **ACR < 30 MG/G OF CREATININE, %** | **ACR > 30 MG/G OF CREATININE, %** |
| I/I                    | 18 (36)                  | 52 (40.3)                | **P = .61**              |
| I/D + D/D              | 32 (64)                  | 77 (59.7)                |

Abbreviations: ACE, angiotensin-converting enzyme; ACR, albumin/creatinine ratio.

| Table 5. Comparison of ACE gene polymorphism among 3 groups of patients. |
|-------------------------|-------------------------|-------------------------|-------------------------|
| **ACE GENOTYPE**       | **ACR < 30 MG/G OF CREATININE, %** | **ACR = 30-300 MG/G OF CREATININE, %** | **ACR > 300 MG/G OF CREATININE, %** |
| I/I                    | 18 (36)                  | 40 (46.0)                | 12 (28.6)                | **P = .29**               |
| I/D                    | 26 (52)                  | 36 (41.4)                | 20 (47.6)                |
| D/D                    | 6 (12)                   | 11 (12.6)                | 10 (23.8)                |
| **Total**              | 50                       | 87                       | 42                       |

Abbreviations: ACE, angiotensin-converting enzyme; ACR, albumin/creatinine ratio.
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