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Angiotensin I-Converting Enzyme Gene Polymorphism in Type 2 Diabetic Patients with Nephropathy

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Angiotensin-I Converting Enzyme (ACE), a major component of the Renin-Angiotensin System (RAS) regulates the renal hemodynamics of our body. An insertion/deletion (I/D) polymorphism of ACE gene has been implicated in kidney disease, hypertension and cardiac function. In the present study, we investigated I/D polymorphism of ACE gene in 125 subjects of both sexes, consisting of 66 normal controls and 59 type 2 diabetic patients with nephropathy. The allele and genotype frequency were significantly different between the patients with diabetic nephropathy and control groups ($p < 0.001$). In the nephropathic patients, ID and DD genotypes were present in 37.2 and 40.6% of patients respectively as compared to 42.4 and 21.2% in those of control groups. The D allele was present in 55.5% of the nephropathic patients and 44.4% of the normal controls. Therefore, percentage of DD genotype and D allele was significantly higher in nephropathic patients compared to normal controls.

Key words: Angiotensin converting enzyme, genotype, allele, nephropathy, polymorphism

INTRODUCTION

Diabetic nephropathy is an important cause of end-stage renal disease secondary to diabetes mellitus. The pathogenesis of this complication is not clearly understood, but available data suggests that multiple factors such as hemodynamic alterations, metabolic abnormalities, various growth factors and genetic factors contribute to this complication (Tarnow, 1996). Genetic predisposition to diabetic nephropathy in patients with type 2 diabetes has been reported (Pettitt *et al.*, 1990; Freedman *et al.*, 1995). Earlier studies have shown that Angiotensin-Converting Enzyme (ACE) inhibitors can improve glucose utilization and suppress hepatic glucose production in patients with type 2 diabetes (Torlone *et al.*, 1991; Jauch *et al.*, 1987). Renin-angiotensin system therefore, may play a critical role in the initiation and progression of diabetic nephropathy (Marre *et al.*, 1994).

The ACE is encoded by a 21 Kb gene that consists of 26 exons and is located on chromosome 17. The major function of ACE is the conversion of angiotensin I to vasoactive, natriuretic octapeptide angiotensin II. The polymorphism of ACE gene involves the insertion (I) or deletion (D) of a 287 bp Alu repeat sequence near the 3 end of intron 16 (Rigat *et al.*, 1992). In type 2 diabetes mellitus patients there are conflicting results regarding the relationship between the ACE genotype and diabetic nephropathy (Schmidt *et al.*, 1995; Mizuiri *et al.*, 1995; Ohno *et al.*, 1996; Smith *et al.*, 1995; Fujisawa *et al.*, 1995; Ha and Seo, 1997). Difference in the frequencies of I/D polymorphism in different ethnic group might be one reason. Therefore, this study was carried out to determine whether ACE gene polymorphism is associated with type 2 diabetes mellitus in the Bangladeshi population and to see whether the association differs from other populations.

MATERIALS AND METHODS

Study subjects: The study included 125 subjects of both sexes, consisting 66 age-matched controls and 59 type 2 diabetic patients with nephropathy. The normal controls were recruited from individuals who had a general health evaluation. Type 2 diabetic patients were selected from Department of Nephrology, Dhaka Medical College Hospital, who were treated as well as ones in periodic follow-ups thereafter. All patients received a baseline examination, blood biochemistry, urinalysis and renal sonography. Diabetic nephropathy was defined as overt proteinuria ($> 500 \text{ mg day}^{-1}$) with or without elevated serum urea and creatinine.

Collection of blood samples: Approximately 3 mL of peripheral blood samples were collected in a screw cap tube that contained 20% EDTA. The specimen was capped and transported to the laboratory under cold condition and stored at -20°C if not analyzed immediately.

Determination of ACE genotype: Genomic DNA was isolated from whole blood according to the published protocols for extracting DNA (Gustincich *et al.*, 1991). To determine the ACE genotype, genomic DNA was amplified by polymerase chain reaction (PCR) initially using a flanking primer pair and subsequently with a primer pair that specifically recognizes the insertion specific sequence, when necessary, in order to confirm the specificity of the amplification reaction (Yoshida *et al.*, 1996). The sense oligonucleotide primer was 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and the antisense primer was 5'-GAT GTG GCC ATC ACA TTC GTC AGA TTT-3'. The PCR reaction mixture contained 50 ng genomic DNA, 10 pmol of each primer, $250 \mu\text{mol L}^{-1}$ dNTP, $1.5 \mu\text{mol L}^{-1}$ MgCl_2 , $10 \mu\text{mol L}^{-1}$ Tris-HCl (pH 8.3) and 1.0 unit Taq DNA polymerase (Invitrogen) in a final volume of 25 μL . The amplification cycle was performed on a 2700 thermal cycler (Applied Biosystems). After initial denaturation at 94°C for 5 min, the DNA was amplified by 35 cycles: denaturation 1 min, annealing at 58°C for 1 min and extension at 72°C for 3 min, followed by a final elongation at 72°C for 5 min. Amplification products were separated by electrophoresis on a 2% agarose gel and visualized under UV light after ethidium bromide staining. The PCR product was a 190 bp fragment in 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 the three possible genotype after electrophoresis: II genotype (a 490 bp band), DD genotype (a 190 bp band), or I/D genotype (presence of both 490 and 190 bp bands (Rigat *et al.*, 1992).

Statistical analysis: Statistical analysis was performed using the statistical package SPSS ver. 11.5. Allele frequencies were deduced from genotype frequencies. The differences in allele and genotype frequencies between the groups were tested by the Chi-square test. The same test was also used to examine if the observed genotype frequencies were in Hardy-Weinberg equilibrium. The association between ACE I/D polymorphism and clinical variables was examined by one-way ANOVA.

RESULTS

As shown in Table 1, the two study groups were well matched for gender and age. The mean Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), fasting

Table 1: Clinical characteristics of control and nephropathic patients with type 2 diabetes

Variables	Control subjects (n = 66)	Nephropathic patients (n = 59)	p-value
Age (years)	47.7±9.87	50.6±10.1	0.891
	35 (53.0)/	32 (54.2)/	0.421
Sex: Male/Female (%)	31 (47.0)	27 (45.8)	
SBP (mm Hg)	120.6±13.4	154.4±19.0	< 0.01
DBP (mm Hg)	79.9±8.01	91.6±12.3	< 0.01
Duration of diabetes (years)	-	10.1±6.87	-
Fasting blood-sugar (mmol L ⁻¹)	5.07±1.00	11.3±4.21	< 0.001
Urea (mg dL ⁻¹)	32.5±11.0	116.0±53.4	< 0.001
Creatinine (mg dL ⁻¹)	0.80±0.18	8.16±3.05	< 0.001

Table 2: Distribution of ACE genotype and allele frequency in the control and nephropathic patients with type 2 diabetes

Variables	Control subjects (n = 66)	Nephropathic patients (n = 59)	p-value
Genotype, No. (%)			
DD	14 (21.2)	24 (40.6)	
ID	28 (42.4)	22 (37.2)	
II	24 (36.3)	13 (22.0)	< 0.001
Alleles			
D	56 (44.4)	70 (55.5)	
I	76 (61.2)	48 (38.9)	< 0.001

blood sugar, serum urea and creatinine was significantly higher in the nephropathic patients than the control groups (p<0.01). The mean duration of diabetes in nephropathic patients were 10.1 years. The ACE genotype distribution in total study subjects, patients and in control groups were in the Hardy-Weinbergs equilibrium ($\chi^2 = 2.512$, df = 2, p = 0.285; $\chi^2 = 3.492$, df = 2, p = 0.175; $\chi^2 = 4.73$, df = 2, p = 0.094).

Table 2 shows the ACE genotype distribution in nephropathic and control groups. Among the nephropathic patients the frequency of the DD, I/D and II genotype were 40.6% (n = 24), 37.2% (n = 22) and 22.0% (n = 13), respectively, as compared to 21.2% (n = 14), 42.4% (n = 28) and 36.3% (n = 24) in control subjects. The frequencies of D and I allele among the nephropathic patients were 55.5 and 38.9% and among the controls they were 44.4 and 61.2%, respectively. Statistical analysis showed that the ACE DD genotype was significantly higher in nephropathic subjects (p≤0.001). The frequency of D allele was more frequent in nephropathic patients than in the control groups (p≤0.001).

DISCUSSION

Diabetic nephropathy is a glomerular disease secondary to diabetes mellitus, accounts for reduced life expectancy. The Angiotensin I-Converting Enzyme (ACE) has been proposed one of the first candidate gene in diabetic nephropathy for several reasons. First, angiotensin II, the final product of the rennin-angiotensin

system, increases intraglomerular pressure (Hall *et al.*, 1977). Second, the glomerular pressure disequilibrium observed in diabetic rats is corrected by ACE inhibitors (Zatz *et al.*, 1986). Third, ACE levels are thought to play a critical role in determining intrarenal angiotensin and kinin corrections (Alhenc-Gelas *et al.*, 1989).

In this study we investigated the association of ACE gene insertion/deletion (I/D) polymorphism with nephropathy in type 2 diabetic patients in a selected population of Bangladesh. We determined the ACE genotype in 125 subjects that consisted 66 healthy controls and 59 type 2 diabetic patients with nephropathy. The two groups were well matched for age and sex but significantly different with respect to SBP and DBP, which is in accordance with one of the previous study conducted in this laboratory (Morshed *et al.*, 2001). As expected, the nephropathic patients had significantly higher levels of fasting blood sugar, serum urea and creatinine.

This study demonstrated a positive association between the D allele (ID and DD genotype) of the ACE gene polymorphism and diabetic nephropathy in the selected population. Several other studies had also found D allele to be an independent risk factor for nephropathy in type 2 diabetic patients (Viswanathan *et al.*, 2001; Doi *et al.*, 1996; Yoshida *et al.*, 1996; Ohno *et al.*, 1996). A meta analysis showed that patients who were homozygous for the deletion allele (DD) had a rapid decline in the renal function and the D allele also appeared to be significantly associated with diabetic nephropathy (Fujisawa *et al.*, 1998). We however, did not observe any association between D allele and the severity of nephropathy. Small numbers in each allelic group might be related to this finding. The result of this study therefore, supports the hypothesis that the DD genotype is in linkage disequilibrium with a functional variant of the ACE gene.

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