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Angiotensin-I Converting Enzyme Polymorphism and Diabetic Nephropathy in North India

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KEYWORDS ACE; I/D polymorphism; diabetic nephropathy; hemodialysis; North Indian population

ABSTRACT Diabetic nephropathy represents a major complication in patients with either type I or type II diabetes. The contribution of a 287 bp insertion/deletion (I/D) polymorphism of the gene encoding angiotensin-I converting enzyme (ACE) has been investigated and the deletion type is documented to be a risk factor in the development of this disease. The present study was designed to determine the relationship between this polymorphism and the risk of developing advanced form of diabetic nephropathy (end stage renal disease) in type II diabetic patients from North India, known to have a high incidence of diabetes and hypertension. Polymerase chain reaction was employed to genotype the DNA isolated from peripheral blood of age and sex matched 117 subjects (59 diabetic nephropathy patients and 58 normal healthy controls). All the subjects, identified as DD, were reconfirmed with an insertion-specific primer. There was no significant difference in the distribution of DD, ID and II genotypes between diabetic nephropathy (10, 32, 17) and normal healthy subjects (9, 33, 16), respectively. The D allele frequencies within patient (0.441) and control (0.440) populations were very similar. The findings of the present study strongly suggest that I/D polymorphism of ACE gene is not implicated in the diabetic nephropathy of North Indian patients.

INTRODUCTION

Diabetic nephropathy is a leading cause of end stage renal disease (ESRD) worldwide and the number of patients needing dialysis or kidney transplantation is increasing steadily (Marshall 2004). Genetic predisposition studies suggest a potential role of genetic factors in the pathogenesis of diabetic nephropathy and the gene encoding angiotensin-I converting enzyme (ACE) is a potential candidate gene in its etiology. ACE, a potent vasoconstrictor, catalyzes the conversion of angiotensin I to angiotensin II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis (Crisan et al. 2000). ACE gene has been described with an insertion/deletion polymorphism (I/D) of a 287 bp *Alu* repetitive sequence in intron 16 (Rigat et al. 1992) leading to three genotypes, DD and II homozygotes and ID heterozygote. The mean plasma/serum ACE level in the DD subjects is reported to be approximately double that of II subjects, with ID subjects having intermediate values (Rigat et al. 1990; Pasha et al. 2002). In a pioneering study, Marre et al. (1994) proposed a protective effect of the II genotype against the development of diabetic nephropathy in insulin-dependent diabetes mellitus. Thereafter, a sizeable number of association studies have investigated the possible role of ACE I/D polymorphism in the pathophysiology of diabetic nephropathy and most of them have recorded association of the D allele as a risk factor (Ng et al. 2005). The Punjabi population from North India is a unique population with a very high incidence of diabetes and hypertension (Gupta et al. 2004). Although, the precise number of patients with ESRD on hemodialysis in India is not known due to the lack of national registries but it is anticipated that around 100,000 patients are added each year to a large group of existing cohort (Sakhuja et al. 2003). It is important to mention that these are conservative estimates as a vast majority of poor patients who can't afford the high cost of renal replacement therapies go unnoticed. The present study was designed to determine the role of ACE polymorphism in Punjabi type II diabetic patients with advanced form of nephropathy undergoing hemodialysis.

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MATERIAL AND METHOD

Study Population: Diabetic nephropathy diagnosis was based on physician's recommen-

dations. Only type II diabetic patients on hemodialysis with advanced irreversible renal failure were selected for the present study. Information related to name, age, sex, family history of the patients was recorded. Informed consent was taken from all the individuals participating in the study. A total of 117 blood samples were collected, in disodium-EDTA, of which 59 samples were from diabetic nephropathy patients visiting Ram Saran Dass Kishori Lal Charitable Trust Hospital, Amritsar, Guru Nanak Dev Hospital, Amritsar and Kidney Hospital, Jalandhar. The rest 58 were age and sex matched normal healthy controls with no history of renal disease, diabetes mellitus and hypertension.

Genotyping ACE I/D polymorphism: DNA was extracted from collected blood samples by modified inorganic method as described by Miller et al. (Miller et al. 1988) and quantified following standard spectrophotometric analysis. Polymerase chain reaction was used to study I/D polymorphism of ACE gene using the published primers (Rigat et al. 1992), flanking a 287 bp insertion sequence. The optimized reaction conditions consisted of 40 ng of genomic DNA in a reaction volume of 30 µl containing 0.16 µM of each primer, 30 µM of each dNTP, 10 µM Tris-HCl (pH 9.0), 1.5 µM $MgCl_2$, 50 µM KCl, 0.01 % gelatin and 0.3 U of Taq DNA polymerase (Bangalore Genei, Bangalore). Amplification was carried out for 35 cycles, each cycle consisting of denaturation at 94°C for 30 s, annealing at 58°C for 20 s, extension at 72°C for 20 s and finally a 3 min extension at 72°C. The PCR products were analyzed in 2% agarose gel and visualized following ethidium bromide staining.

All samples, identified as DD after initial amplification, were reconfirmed with an insertion-specific primer pair, (forward primer 5'-GCCACTACGCCCGGCTAAT-3'; reverse primer 5'-GATGTGGCCATCACATTCGTCAGAT-3'). The reaction conditions and amplification parameters for this confirmatory reaction were the same as stated above. Known controls of each genotype were included with each set of samples for the ACE I/D polymorphism.

Statistical Analysis: Allele frequencies between the diabetic nephropathy and normal healthy control populations were compared using 2x2 contingency tables and chi-squared (χ^2) statistics. Statistical significance was defined at the standard 5% level.

RESULTS

The DNA samples from 59 diabetic nephropathy and 58 normal healthy controls were amplified for I/D polymorphism in the ACE gene and analyzed. Figure 1 represents the PCR products of 190 and 490 bp indicating the presence of deletion (DD) and insertion (II) genotype, respectively. The preferential amplification of the D allele and inefficiency of the amplification of I allele may result in the

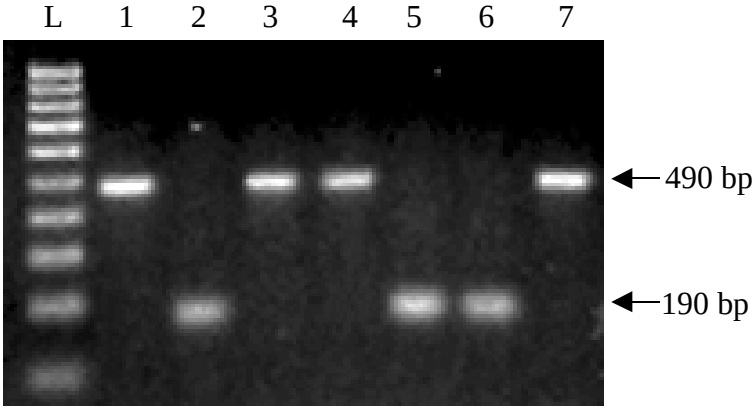

Fig. 1. Agarose gel electrophoresis stained with ethidium bromide, showing the initial amplification for ACE I/D polymorphism. Lane L represents the 100 bp ladder. The II genotype for I allele was identified by the presence of single 490 bp product (Lanes 1, 3, 4 and 7). The DD genotype for D allele was identified by the presence of a single 190 bp product (Lanes 2, 5 and 6). The DD homozygotes were reconfirmed with insertion specific primer pair to avoid mistyping as ID heterozygotes.

mistyping of ID heterozygotes as DD homozygotes. Therefore, in order to increase the specificity of DD genotyping, all samples, identified as DD after initial amplification were reconfirmed with an insertion-specific primer pair, as mentioned in material and method section. The presence of insertion sequence was revealed by the amplification of a 275 bp fragment, while DD homozygotes failed to amplify due to the lack of annealing site (Fig. 2).

Table 1 shows the distribution of ACE genotypes in diabetic nephropathy patients and normal controls. The frequency of D allele and DD genotype was only marginally higher in

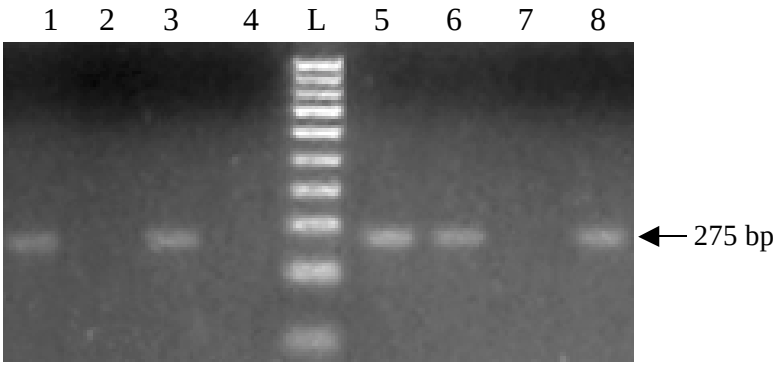

Fig. 2. Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of a product in the lanes 2, 4 and 7 confirms the presence of DD genotype. Heterozygous individuals (ID genotypes) were confirmed by the presence of a single 275 bp product (Lanes 1, 3, 5, 6 and 8). Lane L represents the 100 bp ladder.

Table 1: Distribution of the genotype and allele frequencies in the study groups for the angiotensin converting enzyme (ACE) I/D polymorphism.

Population	*Genotype frequencies (Percentage)*			*Allele frequencies*	
(n)	*DD*	*ID*	*II*	*D allele*	*I allele*
Diabetic nephropathy (59)	10 (17.0%)	32 (54.2%)	17 (28.8%)	0.441	0.559
Normal controls (58)	9 (15.5%)	33 (56.9%)	16 (27.6%)	0.44	0.56

$\div^2$ based on allele frequency [degrees of freedom (df) = 1], (Diabetic nephropathy Vs Controls) = 0.00025; P = 1.000

Table 2: The genotypic distribution and allele frequencies of the ACE I/D polymorphism in different populations of the world.

Population	*Genotype distribution*			*Frequencies*		*Reference*
Studied (n)	*DD*	*ID*	*II*	*D allele*	*I allele*	
North Indian (58)	9	33	16	0.44	0.56	Present Study
Caucasians:						
French (157)	48	69	40	0.525	0.475	Marre et al. (1997)
Czech (302)	87	150	65	0.536	0.464	Hubacek et al. (2000)
Denmark (190)	67	77	46	0.555	0.445	Tarnow et al. (1995)
UK (166)	55	79	32	0.569	0.431	Chowdhury et al. (1996)
German (347)	131	154	62	0.599	0.401	Schmidt et al. (1997)
Boston (77)	16	41	20	0.474	0.526	Doria et al. (1994)
London (85)	24	37	24	0.5	0.5	Powrie et al. (1994)
Asians:						
Japanese (1730)	325	734	671	0.4	0.6	Tamaki et al. (2002)
Chinese (1183)	128	496	559	0.318	0.682	Wang et al. (2005)
Korean (107)	17	41	49	0.351	0.649	Oh et al. (1996)
Tibetan (123)	15	60	48	0.366	0.634	Gesang et al. (2002)
Taiwanese (263)	24	106	133	0.293	0.707	Hsieh et al. (2000)
Turkish (37)	10	22	5	0.57	0.43	Ergen et al. (2004)

diabetic nephropathy patients as compared to the normal controls. The observed and expected genotypic frequencies were in Hardy-Weinberg Equilibrium.

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).

A large variation abounds in the frequencies of ACE I/D polymorphism in different ethnic groups (Table 2). It is evident from this table that the D allele frequency of our controls was intermediate to most reported Caucasian (Marre et al. 1997; Hubacek et al. 2000; Tarnow et al. 1995; Chowdhury et al. 1996; Schmidt et al. 1997) and Asian (Hsieh et al. 2000; Wang et al. 2005; Tamaki et al. 2002; Oh et al. 1996; Gesang et al. 2002; Ergen et al. 2004) populations. However, two Caucasian (Doria et al. 1994; Powrie et al. 1994) and an Asian (Tamaki et al. 2002) population are reported to have comparable allele frequencies.

The findings of Ng et al. in their meta-analyses, encompassing studies conducted between 1994 and 2004, revealed the association of D allele with the risk of having diabetic nephropathy in 14,727 patients from different geographical regions of the world. They further observed that the association was most marked among the Asian type II diabetic nephropathy patients while the Caucasians with the same etiology showed the least association. However, exceptions to such association have also been reported (Oh et al. 1996; Ergen et al. 2004).

People from the Indian subcontinent are at an increased risk of developing diabetes with the incidence among the North Indian population of Punjab being exceptionally high (Gupta et al. 2004). In a recent report, Pasha et al. studied the incidence of ACE polymorphism in different ethnic groups of India. The D allele frequencies in the five population groups from Punjab, Harayana, Himachal Pradesh, Assam and Uttaranchal were 0.511, 0.450, 0.433, 0.423 and 0.409, respectively. Of the five groups only the population from Punjab showed marginally high number of DD homozygotes over the II homozygotes. The failure to find statistically significant differences in the distribution of ACE gene I/D genotypes and their allele frequencies between the diabetic nephropathy patients and the controls (Table 1) suggest that this polymorphism is not a risk factor for the development of diabetic nephropathy in the studied population. Given the high incidence of diabetes and hypertension in Punjabi population it is safe to presume that either there are other genetic and environmental factors than ACE gene that predispose such patients to ESRD or the genes implicated in the etiology of diabetic nephropathy might be masking the effect of ACE gene. These observations find support in the work of Tamaki et al. (2002) and Ergen et al. (2004).

In conclusion, our study suggests that the ACE I/D polymorphism is not associated with advanced form of diabetic nephropathy within the North Indian population.

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