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Distribution of ACE I/D Polymorphism in the Patients of Diabetes and Nephropathy in Pakistan

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ABSTRACT Diabetes mellitus (DM) is a chronic metabolic syndrome that can lead to serious vascular complications. Diabetic nephropathy (DN) has been established as the leading cause of deaths in diabetes due to ESRF. The association between ACE gene polymorphism and onset of DN has not been explored in Pakistani diabetic patients. This study investigates the possible association of insertion (I) and deletion (D) polymorphism of ACE gene in patients of diabetes with and without nephropathy. Total 296 diabetic patients without nephropathy (DM), 168 with nephropathy (DN) and 150 normal healthy individuals were selected followed by informed consent. Fasting blood samples were collected for biochemical analyses and PCR amplification was done to genotype the DNA, for ACE I/D using specific primers. In DM group, the ACE genotypes were distributed as II, 41.55%, DD, 8.45% and ID, 50%. In DN patients, II, 10.71%, DD, 30.95% and ID, 58.33%. The II and DD genotype, and I and D allele distributions were significantly different in DN vs. DM patients (χ^2=9.879, P=0.00167). The I/D genotypes and allele distributions were significantly different in DM patients vs. controls (χ^2=22.252, P=0.00000239). The DN patients have significantly higher prevalence of D allele and DD genotype in comparison to DM. Results indicated a clear association of D allele polymorphism in ACE gene with nephropathy in patients of diabetes. It is suggested that D allele polymorphism may be considered as genetic risk factor and disease marker for nephropathy in diabetes.

INTRODUCTION

Diabetes mellitus (DM) has been considered as the most common cause of morbidity and mortality in various developed as well as developing countries. It may lead to many vascular complications like hypertension, nephropathy, retinopathy, neuropathy and foot infections (Bouchard et al. 2009). Diabetic nephropathy (DN) characterized by persistent albuminuria is a leading cause of end stage renal failure (ESRF). This condition indicates a marked decline in glomerular filtration rate (GFR) and renal fibrosis with loss of function, coupled with hypertension, increased cardiovascular disease risk and eventually ESRF due to progressively rise in urinary albumin excretion rate (UAER). It has been documented worldwide that diabetic kidney disease increases the incidence of ESRF (Marshal et al. 2004). Diabetes and its complications including chronic hyperglycemia, systemic and intra renal hypertension, dyslipidemia, obesity, smoking, aging, high degree of insulin resistance, male sex, race, carbohydrate rich diet and familial clustering of diabetic complications are involved by the number of risk factors direct or indirect, dependent or independent (USRD, 2008). The effect of genetic polymorphism and its role in the development of disease cannot be overlooked. Pathogenesis of diabetic nephropathy is implicated by the polymorphisms in genes encoding the components of renin-angiotensin-aldosteron system (RAAS) which include angiotensinogen, angiotensin-II receptor and particularly angiotensin converting enzyme (ACE) gene (Fogarty et al. 2000). ACE is an important regulatory enzyme and is considered to be a key component of RAAS (Ahluwalia et al. 2009), which catalyzes the C-terminal dipeptide cleavage (His-Leu) from angiotensin-I and converts it to angiotensin-II, which is a potent vasoconstrictor (Eisenmann et al. 2009). It is a pro-inflammatory and pro-oxidant molecule which causes cellular toxicity and apoptosis. It describes that the future risk of impaired glucose tolerance and type 2 diabetes mellitus can be predicted by the chronic low grade systemic inflammation. The regulation of arterial

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pressure, blood volume, cardiac and vascular function, and electrolyte metabolism is mediated by the ACE of the RAAS system (Davis et al. 1997).

Recently, ACE gene has received substantial attention as possible candidate for DM and its complications like hypertension, cardiovascular diseases, and nephropathy (Passaro et al. 2011). ACE gene consists of 26 exons; the amino acid domain of the gene is encoded by exons 1-12 while exons 13-26 encode for the carboxyl domain. The Insertion-deletion (I/D) polymorphism of 287-bp alu repetitive sequences at intron 16 of the ACE gene is the frequently occurring variant which results in 3 different genotypes; II, ID and DD (Freeman et al. 2002). The II and DD are homozygotes and ID is heterozygote. This polymorphism is responsible for the variability in the activity of ACE in serum and various tissues as well as deletion is found to be associated with raised activity of the enzyme (Rigat et al. 1992). On the other hand, the low ACE activity increases insulin stimulated hexose transport in adipocytes and insulin suppression of non-esterified fatty acid flux (Jonsson et al. 1994).

Objectives

This study was designed to investigate the distribution and possible association of ACE gene I/D polymorphism in Pakistani diabetic patients suffering from nephropathy and to identify the prevalent genotype for the development and progression of nephropathy in diabetic patients in Pakistan.

MATERIAL AND METHODS

Study Population

A total of 464 type 2 diabetic patients; 296 without nephropathy (DM) and 168 with nephropathy (DN) were selected followed by informed consent from various hospitals and medical centers of Karachi and Islamabad, Pakistan. The diagnosis of DM was made according to WHO's criteria (Goldman et al.1987). The duration of DM in all the patients was more than 5 years. The diabetic patients on hemodialysis with persistent albuminuria (>300 mg/day) and advanced irreversible renal failure were considered as DN patients. DM patients who had a history of hypertension or renal impairment prior to the development of diabetes and those who with findings suggesting secondary hypertension such as renovascular, renal parenchymal, thyroid or adrenal diseases were excluded from the study. Total 150 age and sex matched, normal healthy control subjects with no known history of hyperglycemia, hypertension, albuminuria or renal insufficiency were also included in the study. The following physiological and clinical variables were measured; Height and weight of patients were measured for identification of body mass index (BMI) using BMI=weight in kilograms/height in squared meters according to Quetelet equation, systolic and diastolic blood pressures by mercury sphygmomanometer, fasting blood glucose by enzymatic colorimetric method, HbA1c by fast ion exchange resin separation method, total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL), triglycerides (TG), serum urea and creatinine by enzymatic colorimetric methods.

ACE I/D Polymorphism Detection

After 12 hours or more fasting, peripheral blood samples from patients and controls were collected. The genomic DNA was extracted from the collected peripheral blood lymphocytes using the standard phenol chloroform extraction method. Polymerase chain reaction (PCR) was used to detect I/D polymorphism of the ACE gene using thermocycler (Eppendorf Master Cycler, USA).

Amplification of the extracted DNA was carried in a final volume of 20ul which contains DNA (250ng), dNTPs (2mmol) (Fermentas), $MgCl_2$ (50mmol), each primer (20µmol), *Taq* polymerase (1.25unit) (5units/µl, Fermentas) in a standard 10X PCR buffer. The flanking primer pair used were 5'-CTGGAGAGCCACTCCCATCCTTTCT-3' as forward primer and 5'-GACGTG GCCATCACATTCGTCAGAT-3'as reverse primer. The cycling conditions for PCR were initial denaturation at 94°C for 1 minute 1 cycle, followed by 35 cycles of 94°C for 1 minute (melting), 58°C for 30 seconds (annealing), 72°C for 1 minute (extension) and final extension at 72°C for 8 minutes. The amplified PCR products were analyzed on 2% agarose gel (w/v) containing ethidium bromide (0.5µg) (Sigma-Aldrich, St. Louis, USA). Agarose gel was visualized using gel documentation system (BioRad, Italy). Three genotypes of ACE gene were iden-

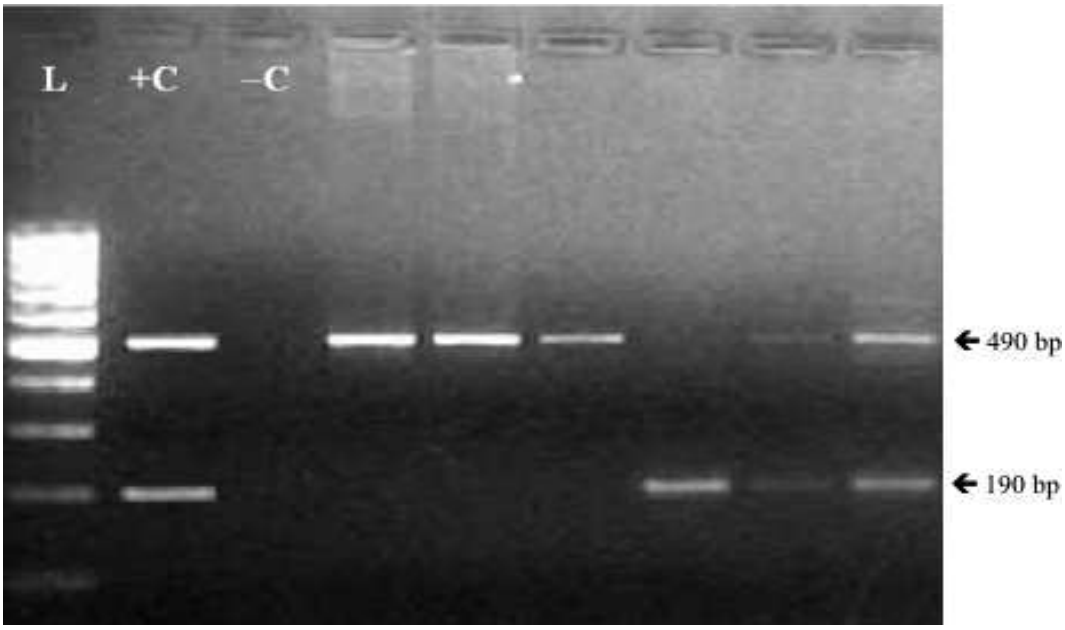

Fig. 1. Agarose gel electrophoresis showing the amplification for ACE I/D Polymorphism; L indicates 100bp DNA ladder, +C: Positive control, -C: Negative control, 490 bp: homozygous II, 490 and 190: Heterozygous ID, 190bp: Homozygous DD

tified after electrophoresis, 490bp homozygous for insertion alleles (I/I), 490 and 190bp heterozygous for insertion and deletion allele (I/D) and 190bp homozygous for the deletion allele (D/D) (Fig. 1).

Statistical Analysis

The statistical analysis was performed using SPSS statistical package version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA) for Windows. Results were presented as mean± SEM. Statistical comparisons between study groups means were done by Student's *t*-test. Genotypic and allelic frequencies of ACE gene were compared in the study groups by Yates' corrected chi-squared test (χ^2). The odds ratio (OR), together with the 95% confidence interval (CI), comparing the allelic distribution in the study groups were also calculated. Statistical significance was defined as standard 5% level ($p<0.05$).

RESULTS

As indicated in Table 1, the study groups of patients were well matched for age, gender, duration of diabetes and BMI. The BMI values of DM group were significantly higher ($p<0.05$) as compared to control. DN group showed significantly higher ($p<0.05$) levels of SBP, DBP, HbA1c cholesterol, LDL, TG, urea and creatinine values as compared to control. On the other hand, the level of HDL in DN was significantly low ($p<0.05$) as compared to control. DM as well as DN patients showed significantly higher ($p<0.05$) fasting blood glucose levels as compared to controls (Table 1).

The genotypic and allelic frequencies of the ACE gene I/D polymorphism in groups of patients and controls are given in Table 2. The ACE genotype were distributed as II, 18 (10.71%); DD, 52 (30.95%) and ID, 98 (58.33%) in DN patients. The DM patients represented II, 123 (41.55%; DD, 25 (8.45%) and ID, 148 (50%). The controls showed II, 42 (28%); DD, 60 (40%) and ID, 48 (32%). It identifies that the D allele distribution is significantly higher in patients of persistent nephropathy (DN) group (60%) as compared to diabetes without nephropathy (DM) group (33.45%) (Table 2).

The genotypic (II and DD) and allelic (I and D) frequency distributions in the study groups were also compared and presented in Table 3.

Table 1: Characteristics of controls, DM and DN patients

Parameters	*Controls n=150*	*DM group n=296*	*DN group n=168*
Age (years)	55.93± 8.43	56.58±10.95	57.62± 9.07
Sex (M/F)	82/68	150/146	90/78
Diabetic age (years)	NA	15.6 ± 7.49	16.44± 6.97
BMI (Kg/m^2)	26.9 ± 6.58	27.54± 3.22*	26.85± 5.31
SBP (mm Hg)	123.25± 4.85	144.5 ±10.76*	153.24±12.44*$^+$
DBP (mm Hg)	79.73± 6.98	81.82± 8.91*	98.71± 9.67*$^+$
FBS (mmol/L)	5.38± 1.45	8.53± 4.5*	11.22± 5.26*$^+$
HbA1c (%)	NA	9.52± 2.44	12.24± 4.37$^+$
Cholesterol (mmol/L)	4.84± 0.57	6.74± 0.88*	7.64± 2.45*
LDL (mmol/L)	2.57± 0.43	2.92± 0.46	3.4 ± 0.41*$^+$
HDL (mmol/L)	1.38± 0.19	1.06± 0.12	0.88± 0.14*$^+$
TG (mmol/L)	0.99± 0.18	1.02± 0.23*	1.42± 0.22*$^+$
Urea (mmol/L)	11.46± 2.22	9.62± 2.01	19.36± 4.58*$^+$
Creatinine (µmol/L)	113.39±22.59	115.52±32.59	198.44±26.41*$^+$

Values are mean±SEM,
*p<0.05 as compared to controls, +p<0.05 as compared to DM group

Table 2: Distribution of ACE genotypes and allele frequencies in DN, DM and control groups.

Study Groups	*ACE Genotype*			*ACE Allele Frequencies*	
	II	*DD*	*ID*	*I*	*D*
Diabetic Nephropathy (DN)(N=168)	18 (10.71%)	52 (30.95%)	98 (58.33%)	0.4	0.6
Diabetes mellitus without Nephropathy (DM)(N=296)	123 (41.55%)	25 (8.45%)	148 (50.00%)	0.67	0.33
Control(N=150)	42 (28.0%)	60 (40.00%)	48 (32.00%)	0.44	0.56

The II and DD genotype and I and D allele distributions were significantly different in DN patients as compared to DM patients (^{2}=9.897, OR=0.3799, 95% CI=0.2119-0.6811, P=0.00167). No significant difference was found between ACE genotypes and allele frequencies in DN patients when compared with controls (c^2=0.835, OR=2.4093, 95% CI=1.346-4.3125, P=0.3608). Significantly different II and DD genotypes and I and D allele distributions were observed in DM patients as compared to controls (c^2=22.252, OR=3.6585, 95% CI=2.1436-6.2441, P=0.00000239) (Table 3).

DISCUSSION

Diabetes mellitus and its vascular complications are major health concern which progressively increasing burden in most developing economical countries like Pakistan. The incidence of diabetes mellitus is increasing 170% in developing countries as compared to 47% in developed countries (Batzer et al. 1996). Main reasons are population growth, ageing, urbanization, increased prevalence of obesity and physical inactivity (Alberti et al. 1998). Pakistan stands on number 6 among the top ten countries having increased burden of diabetes mellitus. Insufficient data is available regarding prevalence of diabetes in Pakistan (WHO 2003).

Diabetic nephropathy and I/D polymorphism of ACE gene association have not been studied in Pakistani population. The present study describes the association between ACE I/D polymorphism and patients of Diabetes mellitus and diabetic nephropathy of Pakistani patients. In this study, the distribution of D allele (DD homozygous and ID heterozygous) is signifi-

Table 3: Comparison of genotype (II and DD) and allelic (I and D) frequency distribution in study groups

Study Groups	*Yate's Chi-square value*	*Degree of Freedom*	*p-Value*	*Odds Ratio*	*95% Confidence Interval*
DN vs. DM	9.879	1	< 0.050 (.00167)	0.3799	0.2119-0.6811
DN vs. Controls	0.835	1	> 0.050 (.3608)	2.4093	1.346 -4.3125
DM vs. Controls	22.252	1	< 0.050 (.00000239)	3.6585	2.1436-6.2441

cantly higher in patients of persistent nephropathy (60%) as compared to diabetes without nephropathy (33.33%). Similarly, the DD genotype was found in 30.95% and II genotype was found in 10.71% patients of nephropathy with diabetes. The patients of diabetes suffering from nephropathy showed contradictory distribution of D allele from different regions of the world. An Indian study with approximately 100 patients and controls reported no significant association of ACE I/D polymorphism with nephropathy (Anbazhagan et al. 2009). In the specific case of diabetic nephropathy a significantly increased distribution of D allele and the DD genotype was observed in Asian patients compared with diabetic patients without nephropathy and was associated with increased risk of nephropathy (Saab et al. 2007). In South Indian study D allele and severity of nephropathy in diabetes shows no significant association (Viswanathan et al. 2001). A meta-analysis of prevalence reports indicated the overall frequency of D allele is 54% with ethnic differences. In Arab populations (Egyptians, Jordanians and Syrians) for example, frequencies of the D allele are approximately 65%. Although there is evidence that an increased ACE activity associated with the D allele does not result in increased synthesis of angiotensin II, while increased incidence of cardiovascular disease and resistance of ACE inhibitor therapy might be associated with it (Gard et al. 2010).

On the basis of findings, this study helps to understand the ACE I/D genotype and allelic frequencies distribution in diabetic patients with and without nephropathy in Pakistani population. Although, there was no significant difference observed between I and D allele in diabetic nephropathy patients as compared to normal controls, a significantly higher prevalence of D allele was observed in diabetic nephropathy as compared to diabetic patients without nephropathy. On the other hand, a high prevalence of I allele (67%) as compared to D allele (33%) was found in patients of diabetes without nephropathy, indicating that I allele is protective in diabetic patients without nephropathy and II genotype is less prone to develop nephropathy during the course of diabetes mellitus.

CONCLUSION

It can be concluded that the D allele carries with an increased risk of developing nephropathy in diabetic patients of Pakistan. On the positive side, the D allele as a genetic risk marker may be responsible for renal impairments in diabetic patients. However, the association of ACE DD genotype and D allele with diabetic nephropathy is based on a limited number of patients. A more extensive analysis is required to authenticate this study, which will provide possible molecular diagnostic and therapeutic implications and will lead to novel strategies for the better understanding and beforehand treatment of renal insufficiency in diabetes mellitus.

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