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Gender Based Disparities in ACE I/D Polymorphism Associated with Progression of Diabetic Nephropathy in Pakistani Patients with Type 2 Diabetes Mellitus

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Abstract
Type 2 diabetes mellitus (T2DM) has become a global problem. Type 2 diabetes mellitus is accelerating pandemic in the Pakistani population as well. The problem progresses with severity of diabetic complications. Diabetic nephropathy (DN) is the most fatal of all these complications. Diabetic nephropathy leads to end stage renal disease if glycemic control and microvascular protection is inadequate. Angiotensin converting enzyme (ACE) gene polymorphism has been reported to be associated with the diabetic nephropathy in type 1 and type 2 diabetes mellitus. ACE plays crucial roles in regulation of rennin angiotensin system. Use of ACE inhibitors and angiotensin receptor blockers (ARBs) can prevent diabetic renal damage. The purpose of this study is to investigate the association of Insertion/deletion (I/D) polymorphism of 287 bp *Alu* repetitive sequence in intron 16 of ACE gene with diabetic nephropathy. The study includes 284 T2DM Pakistani patients out of which 84 patients developed DN and 108 control subjects. Gender specific ACE gene association was observed in this study. Insertion (I) allele of ACE gene was found to be associated with progression of DN in men with type 2 diabetes mellitus. We did not get any significant association of I allele in women with DN. Neuropathy and family history were strongly associated with progression of diabetic nephropathy. The study will help administer T2DM patients with diabetic nephropathy in Pakistani ethnic groups. Prospective studies need to determine the role of ACE gene I/D polymorphism in Pakistani men with diabetic nephropathy.

Key words: *Type 2 Diabetes Mellitus, Diabetic Nephropathy, Angiotensin converting enzyme, Renin Angiotensin System*

Introduction

Type 2 Diabetes mellitus (T2DM) is an alarmingly accelerating pandemic in the urbanized world. It is posing huge burden on health subjects and poor economies of third world countries including Pakistan. The number of new cases of T2DM patients has been increasing in primary care hospitals of Pakistan for the last two decades. Many of these patients present with diabetic complication like diabetic nephropathy, neuropathy, disturbed lipid profile and gangrene (few patients had been amputated). Two hundred and eighty four T2DM patients were collected for this study. Eighty-four of the patients developed diabetic nephropathy (DN).

DN is one of the most severe microvascular complications developing in T2DM patients. DN is a syndrome of microalbuminuria, relentless loss of glomerular filtration rate (GFR), enhanced cardiovascular threat and hypertension. It affects one third of type 1 and type 2 diabetes mellitus patients.[1-4] Diabetes increases the risk of end stage renal disease approximately 12-times.[5] Diabetes mellitus patients are in a general state of chronic hyperglycemia. This state affects glucose-reliant processes, inciting the pathogenesis of microvascular/macrovascular complications and DN. DN progresses through incipient nephropathy (microalbuminuria) to overt nephropathy, which often necessitates dialysis and renal transplants.[6] The precise pathogenesis of diabetic nephropathy is not fully known. The annotated data suggests factors like haemodynamic shifts, metabolic irregularities, growth factors and genetic factors may cause the complication.[7]

Renin Angiotensin system (RAS) plays a crucial role in physiology and pathophysiology of the kidney. ACE is the regulator enzyme in RAS. It converts angiotensin I to angiotensin II and inactivates bradykinin and kallidin. Angiotensin II is a vasoconstrictor whereas bradykinin and kallidin are vasodilators. The activation of ACE results in vasoconstriction.[8]

ACE gene covers 21 kb on chromosome 17 carrying 26 exons. Based on the insertion (I allele) or deletion (D allele) of 287bp *Alu* repetitive sequence in intron 16 there are three types of polymorphism in the ACE gene; II, DD homozygotes and ID heterozygote. This polymorphism defines the tissue/serum ACE activity and determines the serum/plasma ACE levels, DD genotype individuals have approx. double ACE plasma/serum levels than II genotype individuals and ID individuals have intermediate values.[9-11]

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Previous studies have shown that there are differences in the I/D polymorphism in different ethnic groups.[12,13] T2DM patients with DD ACE genotype have high blood glucose levels and show more glucose intolerance.[14] ACE gene polymorphism has been found associated with diabetic nephropathy in both type 1 and type 2 Diabetes Mellitus. D allele and DD genotype of ACE gene has been reported to be associated with Diabetic nephropathy in T2DM.[15-17] Chronic renal deterioration progresses more rapidly in men than in women.[18] There is increased risk of renal failure of different etiologies in men than in women.[19] Female diabetes patients with D allele of ACE gene are likely to progress diabetic nephropathy.[20]

For renal protection in T2DM patients, angiotensin receptor blockers (ARBs) are administered.[21] ACE inhibitors and ARBs combination has an additive effect in antihypertensive and antiproteinuric therapy.[22] A protective effect of ACE inhibitors is to lower the glomerular systemic hypertension. [23,24]

Materials and Methods

The study was approved by the Institutional ethical committee. Peripheral blood samples from T2DM patients (n=284) and controls (n=108) were collected in Acid Citrate Dextrose vacutainers (BD Franklin Lakes NJ USA) with informed consent. Patients with T2DM were ascertained by laboratory findings of HbA1c, length of receiving treatments for T2DM, the period of being diabetic and the age of onset of diabetes. T2DM with diabetic nephropathy were screened by microalbuminuria, 24 hours urine for protein urea, patients undergoing dialysis, and urea/creatinine levels.

DNA was isolated from peripheral blood lymphocytes of the patients' blood samples using standard phenol extraction protocol.[25] ACE I/D polymorphism was determined by polymerase chain reaction using the gene intron specific primers.[26] Amplification was done in a final volume of 15µl containing 20ng genomic DNA, 1X PCR buffer *(Bioline)*, 0.45mM $MgCl_2$, 200µM dNTPs *(Promega)* 1µM each forward and reverse primer, 1U Taq DNA polymerase (*Institute of Biomedical and Genetic Engineering, Islamabad, PK*). Amplification was done with initial denaturation at 94^{o}C for 3 min, 35 cycles each consisting of denaturation at 94^{o}C for 45 s, annealing at 58^{o}C for 45 s, extension at 72^{o}C for 45 s and final extension for 10 min at 72^{o}C.

Sense primer 5'-CTGGAGACCACTCCCATCCTTTCT-3' and antisense primer 5'GATGTGGC CATCACATTCGTCAGAT-3' were used for amplification. Amplified PCR product was run on 2% (w/v) agarose gel containing 0.5µg/ml ethidium bromide at constant power supply of 200 volts. I allele was identified as 490bp band and D allele as 190bp band by UV transillumination *(Syngene, Cambridge, UK)*; size depicted by 100bp ladder *(Promega)*. Statistical analysis was done by SPSS for windows, version 10.0 (SPSS Inc., Chicago, USA) and Preacher et al. calculator.[27]

Results

In this study, we found the frequency of D allele slightly lower in patients with diabetic nephropathy than in T2DM patients without diabetic nephropathy and controls (Table 1).

For diabetic nephropathy and T2DM subjects odds ratio (OR) value is 1.039 with 95 % with Confidence Interval (CI) 0.7206-1.4981, P value is 0.74. For diabetic nephropathy and control subjects OR value is 1.1695 with 95 % CI 0.7778-1.7585 and P value is 1. These results did not show any significant association of the D allele with Diabetic Nephropathy in T2DM patients. P values are shown in Table 2.

An interesting result is found when ACE I/D polymorphism was analyzed for male and female subjects separately. In males I allele has a significantly greater frequency in Diabetic Nephropathy subjects than observed in controls with OR value 1.9504 with 95 % CI 0.9891-3.8462 and P value 0.05. Analysis of diabetic nephropathy and T2DM without diabetic nephropathy subjects showed OR value 1.8747 with 95 % CI 0.9386-3.7443 and P value 0.07. These results show significant association of I allele with development of diabetic nephropathy in males with T2DM. The P values and allele frequencies for male subjects are shown in table 2 and 3 respectively.

In female subjects, I and D allele frequencies have no significant difference in diabetic nephropathy, T2DM without diabetic nephropathy and controls subjects. Analyses of Diabetic Nephropathy and controls have OR value 1.009 with 95% CI 0.5438-1.872 and P value 0.84 while diabetic nephropathy and T2DM without diabetic nephropathy subjects have OR value 0.8117 with 95 % CI 0.5236-1.2583 and P value 0.35. These results do not show any significant association of ACE I/ D polymorphism with diabetic nephropathy in female subjects. P values and allele frequencies and are shown in Tables 2 and 3 respectively.

Positive family history of patient for T2DM was significantly associated with development of diabetic nephropathy P value 0.004. Neuropathy in Diabetic patients was also strongly associated with diabetic nephropathy P value 0.00012 (Table 4). More over prolonged period of uncontrolled diabetes mellitus is another important contributor in the development of diabetic nephropathy.

Discussion

Nephrology is striving to overcome the high rate of morbidity and mortality of Diabetic Nephropathy. Diabetic Nephropathy is a complex pathophysiological process involving various cellular and molecular mechanisms. Diabetic nephropathy leads to end stage renal disease if it is not managed properly. The most powerful agents for regulating end stage renal disease are hypertension and diabetes mellitus.[21] Variations in the repetitive DNA sequences among different ethnic groups are the genetic factors that are believed to pedestal progression of diabetic nephropathy and end stage renal disease in diabetes mellitus.

Table 1: ACE I/D polymorphism distribution and allele frequencies among male subjects from three groups

Group Studied	Genotypes			Allele frequencies	
	II	DD	ID	I Allele	D Allele
Diabetic Nephropathy Patients (N=84)	27 (32.3%)	12 (14.3%)	45 (53.6%)	0.59	0.41
Type 2 Diabetes Mellitus patients without Nephropathy (N=200)	65 (32.5%)	33 (16.5%)	102 (51.0%)	0.58	0.42
Controls (N=108)	32 (29.6%)	21 (19.4%)	55 (50.9%)	0.55	0.45

Table 2: P values for Diabetic Nephropathy patients, Type 2 Diabetes Mellitus patients and Controls for males, females and total subjects studied.

Group Studied	P values for ACE I allele		
	Males	Females	Total subjects studied
Diabetic Nephropathy patients and patients with Type 2 Diabetes Mellitus without Nephropathy	0.07	0.35	0.74
Diabetic Nephropathy patients and Controls	0.05	0.84	1
Patients with Type 2 Diabetes Mellitus without Nephropathy and Controls	0.87	0.61	0.73

Table 3: ACE I/D polymorphism distribution and allele frequencies among male and female subjects from three groups

Sex	Group Studied	Genotypes			Allele Frequencies	
		II	DD	ID	I	D
Male	Diabetic Nephropathy patients (n=26)	13 (50.0%)	2 (7.7%)	11 (42.3%)	0.71	0.29
	Type 2 Diabetes Mellitus patients without Diabetic Nephropathy (n=66)	18 (27.3%)	9(13.6%)	39 (59.1%)	0.57	0.43
	Controls (n=77)	24 (31.2%)	15 (19.5%)	38 (49.4%)	0.56	0.44
Female	Diabetic Nephropathy patients (n=58)	14 (24.1%)	10 (17.2%)	34 (58.6%)	0.53	0.47
	Type 2 Diabetes Mellitus patients without Diabetic Nephropathy (n=134)	47 (35.1%)	24 (17.9%)	63 (47.0%)	0.59	0.41
	Controls (n=31)	8 (25.8%)	6 (19.4%)	17 (54.8%)	0.52	0.48

Table 4: Comparison of different parameters b/w Diabetic Nephropathy patients and Type 2 Diabetes Mellitus

Parameters	Diabetic Nephropathy Patients (84)	Controls (200)	p Value
Age (yrs)	53.36± 9.71	53.97± 10.17	
Age at DM onset	41.73± 10.50	47.10± 10.05	
BMI (kg/m^2)	27.1231± 4.3054	27.63± 4.8682	
Male	26 (31%)	66 (33%)	0.74
female	58 (69%)	134 (67%)	0.74
Family history	42 (50%)	64 (32%)	0.004
Cardiovascular Disorder	17 (20.2%)	32 (16%)	0.38
Hypertension	24 (28.6%)	51 (25.5%)	0.59
Neuropathy	41 (48.2%)	51 (25.5%)	0.00012

In this study ACE gene ID genotype is frequently found in diabetic nephropathy than II genotype but no significant difference in type 2 diabetes mellitus and control subjects was observed. A meta-analysis study comprising 14,727 subjects showed that II genotypes are at a decreased risk of Diabetic Nephropathy than the D allele carriers in

T2DM.[15,28] We did not find any significant association of D allele in increasing the risk of Diabetic Nephropathy in T2DM in our cohort study.

Gender affect of ACE I/D polymorphism has been observed in this study. I allele has been found significantly associated with progression of diabetic nephropathy in males with T2DM, P value=0.05 (Table 2). This gender-based difference screens the fact that there are differences in Renin Angiotensin system of men and women. Sex steroids are possibly interacting with rennin angiotensin system to generate these gender differences in renal deterioration in diabetes patients. Furthermore chronic kidney disease is likely to develop and progress to end-stage-renal disease more in men than in women based on all-cause incidence rates.[29] Men may need longer dosage of angiotensin receptor blockers for Renin Angiotensin system blockade in kidney.[30]

This study suggests the involvement of ACE I/D polymorphism in progression of diabetic nephropathy. The men with the II genotype being are at an increased risk to progress the diabetic nephropathy. In both men and women, positive family history and neuropathy are major accelerators of diabetic nephropathy in T2DM. This study implies to use ACE inhibitors and Angiotensin Receptor Blockers for rennin angiotensin system blockade in patients, particularly men, with type 2 diabetes mellitus at an early stage of diabetes mellitus. Prospective studies need to investigate the exact mechanism of ACE II genotype role in diabetic nephropathy in Pakistani men. Exploring the possible role of neuropathy as a risk factor for diabetic nephropathy in both men and women is also warranted.

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