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Studies on Association of ACE Gene-1 Insertion/Deletion Polymorphism with Renal Failures in Vysya (Settiyar) Population of Andhra Pradesh, India, a Community Based Study

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Abstract: The present study has been carried out at selective areas of Prakasam and Nellore districts of Andhra Pradesh to evaluate the association between ACE gene I/D polymorphism and progression of renal damage in the Settiyar community. The contribution of insertion/ deletion (I/D) polymorphism of the gene encoding ACE has been investigated and the deletion type is documented to be a risk factor in the development of this disease. 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 renal failure and normal healthy subjects. The findings of the present study state that the frequency of D allele and DD genotype was only marginally higher in both KD and NDD patients as compared to the normal controls. The observed and expected genotypic frequencies were in Hardy-Weinberg Equilibrium.

Key words: Diabetic Nephropathy · ACE Polymorphism · Obesity and Settiyar Community

INTRODUCTION

Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nephrotic syndrome and diffuse glomerulosclerosis. It is due to longstanding diabetes mellitus and is a prime indication for dialysis in many Western countries.

Racial differences in the prevalence of diabetic renal disease have been reported. Asian subjects have significantly ($p<0.01$) higher prevalence (52.6%) of diabetic end stage renal disease (ESRD) when compared with the Caucasians (36.2%) migrant Asians [1]. Indians had 40 times greater risk of developing ESRD when compared with the Caucasians [2]. The prevalence of diabetic nephropathy in type 2 diabetic subjects is reported to be 5-9% from various Indian studies [3-5]. Patients with diabetic nephropathy, especially with type 2 diabetes, have a high cardiovascular risk. The risk for cardiovascular disease (CVD) was 3 fold higher in South Indian NIDDM subjects with nephropathy when compared with their non-nephropathic counterparts [6]. Thus, in type 2 diabetes, many patients may not reach end stage renal disease due to premature death from CVD.

Obesity is the main cause for several life threatening diseases like diabetes. Particularly in countries like India, where various forms of people live with a unity in diversity. Particularly some communities were more susceptible to diseases like diabetes due to their life style and habituates. In Andhra Pradesh, specifically the Vysya community (Settiyar) is more prone towards obesity due to their lifestyle. People belonging to this community are frequently suffering from diabetes and renal failures [7]. Renal failure is an outcome of complex pathophysiological process resulting from multiple etiologies with contribution from both genetic and environmental factors. In recent years a vast amount of data has been published on the association between the insertion/deletion (I/D) polymorphism of the gene coding for angiotensin- converting enzyme and renal disease. It has become clear that the polymorphism does not affect the prevalence of renal disease.

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However, data on the association with progression of renal disease and therapy response are still contradictory. Moreover, sufficient data on the physiological significance of this polymorphism are still lacking. Hence the present study was undertaken to study the association of Ace gene 1 I/D polymorphism with the progression of renal failures in the selected community.

MATERIALS AND METHODS

A study was conducted in Nellore and Prakasam districts of Andhra Pradesh which is geographically southern part of India near to the Bay of Bengal and these districts having wide spread of selected community. The lifestyle and from the reports of local medical laboratories made us to carry out the present study.

Selection of Patients: Eight hundred and twenty (n=820) type II diabetic subjects from 200 families of Shettiyar (Vysya) community in Nellore and Prakasam districts of Andhra Pradesh State were chosen randomly for the present study. A door to door survey with face-to-face interviews was carried out in the same community group to find out the known diabetic (KD) and newly diagnosed diabetic (NDD) subjects. The information collected was entered on a pre-coded questionnaire. Among the total number of samples 200 were KD subjects and remaining 620 were NDD. People suffering from regular renal failure with diabetes and newly diagnosed diabetic were separated.

Collection of Blood and Urine Samples: This study was conducted on around 200 families, who were suffering from renal problems associated with diabetes with KD and NDD. They were provided with explanations for all experimental procedures and informed consent was obtained before the beginning of the study. Blood and urine samples were collected from the subjects and preceded for further hematological and biochemical analysis.

Biochemical Analysis

Estimation of Random Blood Sugar: Random blood glucose was measured routinely by using "One Touch Ultra Blood Glucose Meter" (Accu Chek Gluco Meter, USA).

Beta 2 Microglobulin Assay: The samples were analyzed by using Enzyme linked immunosorbent assay (B2-microglobulin EIA kit, Immunotech, France). 2.4mg/L was used as upper limit, when 97% of normal values are below this cutoff value.

Estimation of Serum and Urinary ACE Levels: Serum or urine Ace levels were measured by a colorimetric method (Colorimetric assay Kit, Fujizoki assay, Tokyo, Japan) using p- hydroxyhippuryl L-histdyl-L-leucine as the substrate [8].

Determination of ACE Genotypes: The D and I alleles were identified on the basis of polymerase chain reaction (PCR) amplification of the respective fragments from intron 16 of the ACE gene and size fractionation and visualization by electrophoresis. DNA was extracted from peripheral leukocytes with standard techniques. PCR was performed with 20 pmoles of each primer: sense oligo 5'CTGGAGACCACTCCCATCCTTTCT3' and anti-sense oligo: 5'GATGTGGCCATCACATTCGTCAGAT3' in a final volume of 25 µl,containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.2mM of each dNTP and 1.25 unit of Taq polymerase (Perkin Elmer-Cetus,Norwalk, CT). The DNA was amplified for 30 cycles with denaturationat 94°C for 30 s, annealing at 58°C for 30 s and extension at72 °C for 1 min, followed by final extension at 72°C for 5min (DNA Thermal Cycler 480, Perkin Elmer-Cetus) (9,10). PCR products were electrophoresed in 2% agarose-gel with 5 µg ethidium bromide per milliliter. The amplification products of the D and I alleles were identified by 300-nm ultraviolet trans-illumination as distinct bands (D allele: 191 bp; I allele: 478 bp) Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (hace 5a, 5'TGGGACCACAGCGCCCGCCACTAC3';hace 5c, 5' TCGCCAGCCCTCCCA TGCCCATAA3'), with identical PCR conditions except for an annealing temperature of 67 °C. The reaction yields a 335-bp amplicon only in the presence of an I allele and no product in samples homozygous for DD [11, 12].

Statistical Analysis: Statistical work was carried out by using SPSS software for Windows 10.0 (SPSS Inc., Chicago, Il, USA) and Microsoft Excel. Values were reported as mean+/-standard deviation. SD was not more than 10%.

RESULTS AND DISCUSSION

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) worldwide and it is estimated that ~20% of type 2 diabetic patients reach ESRD during their lifetime [13]. Microalbuminuria is the earliest clinically detectable stage of diabetic kidney disease at which appropriate interventions can retard, or reverse

Table 1: Comparison of Glucose levels in Control and Diabetic subjects of Selected community

		Random Blood Sugar (mg/dL)	
Subjects		Fasting	PP
Normal value		70-100 mg/L	<150 mg/L
Control (n=200)		83.58±15.83	133.58±5.21
Shettiyar community	KD (n=200)	138.59±18.06	228.32±7.54
	NDD (n=620)	142.21±10.87	239.47±6.12
Significance		P<0.001	P<0.001

the progress of the disease. As hyperglycemia is the preliminary biomarker for the identification of diabetes and developing disease progression, studies were explored in the selected populations under fasting and postprandial visits in terms of blood tests. Results showed that the selected community is having higher glucose levels of 138.59±18.06 mg/dL fasting and 228.32±7.54 mg/dL of postprandial glucose levels, stating that the selected subjects were belongs to diabetic (Table 1). These people considered as KD (n=200), where already they were identified and under treatment category. The other 620 patients were newly discovered that they are diabetic. Hence they were considered as NDD. Results showed that 142.21±10.87 mg/dL of fasting glucose and 239.47±6.12 mg/dL postprandial glucose levels are observed in the NDD category. These results were matched with their family history and life style (Table 1).

The routine classical evaluation of nephropathy (any type of renal problems) includes the identification of glomerular and tubular markers in the patient's serum and urine. The normal individual doesn't contain this content elevated in their urine or in serum samples. These glomerular and tubular markers include: transferrin, IgG, antitrypsin, β-2-microglobulin and angiotensin converting enzyme (ACE). Recent studies also have demonstrated that, there were tubular components in renal complications of disease conditions as shown by the detection of renal tubular enzymes and low molecular weight proteins in the urine as well as in serum. In fact, tubular involvement may precede glomerular involvement because several of these tubular proteins and enzymes are detectable even before the appearance of microalbuminuria and rise in serum creatinine [14].

Thus studies were conducted to evaluate the glomerular and tubular marker in urine as well as in serum of the control and selected community (Shettiyar) people. B2M can be an early marker to diagnose renal failure under fluoride toxicity [15] and other heavy metal toxicity and also under nicotine toxicity [16]. In our study interestingly rapid enhancement of serum B2M was noticed. The control subjects showed 2.60±0.99 g/ml, where the KD people showed a maximum increase of B2M to 10.89±2.08 g/ml and NDD people showed 11.56±1.88 g/ml. That shows a drastic increase which indicates the altered renal activity in the selected group of people. A significant (P<0.001) change was noticed compared to the normal. This altered range is more supportive for further analysis for the diabetic nephropathy in Shettiyar community. Angiotensin-converting enzyme and ACE2 are highly expressed in the kidney. The role of ACE in the development of renal damage is generally accepted. Here in the present study the serum ACE level seems to be decreased when compared to that of control individuals (Table 2). Control individuals had a concentration of 44.97±8.72 and selected group of people belongs to KD showed 37.07±12.68 and NDD people showed a concentration of 32.51±10.23 indicating a significant (P<0.001) decrease. This indicates the accumulation of angiotensin I.

When the above markers were observed in urine B2M also showing similar pattern of over excretion. Here we can find 3.64±0.97 in the KD people and 4.21±0.66 in NDD people, where the control value is 1.24±0.98. Hence, it can be concluded that, these values are drastically increased in the serum as well as in urine of the selected community (Shettiyar) people. The same was also found with ACE levels here the control value is 11.46±0.84 and the KD people are showing 13.77±1.46 and the NDD are showing 16.74±0.89, which means over excretion indicates the renal problems (Table 2).

B2M is normally cleared by the kidneys at a rate comparable to GFR and then reabsorbed and catabolized in the tubules and serum levels are inversely related to GFR [17]. Clearance by conventional dialyzers is negligible as these membranes are impermeable to β2m. Production of β2m in normal is 9 mg/ hr/70 kg [18]. Production may be increased in proliferative disorders [19] and rheumatoid arthritis [20] as indicated by high serum levels in the presence of normal renal function.

Angiotensin-converting enzyme (ACE) is a risk factor for DN. Its plasma levels have been reported to be associated with DN but not with diabetic retinopathy in type 1 diabetes patients [21]. ACE modulates the generation of angiotensin II, which increases intraglomerular hydraulic pressure [22], leading to glomerulopathy. ACE inhibition strongly modifies renal hemodynamics in animals [23] and the course of DN can be considerably improved by treatment with ACE inhibitors, in patients with type 1 diabetes [24]. Plasma ACE concentrations are stable in individuals [25] and are partly under genetic control [26].

Table 2: Comparison of Renal biochemical markers levels in Control and Diabetic subjects of selected community

Subjects selected		Beta-2 MG (serum)	ACE (serum)	Beta-2 MG (urine)	ACE (urine)
Control		2.60±0.99	44.97±8.72	1.24±0.98	11.46±0.84
Selected community	KD (n=408)	10.89±2.08	1132.89±224.93	3.64±0.97	13.77±1.46
	NDD (n=412)	11.56±1.88	1089.24±215.41	4.21±0.66	16.74±0.89
SEM		0.400	2.441	0.183	0.276
Significance		P<0.001	P<0.001	P<0.001	P<0.001

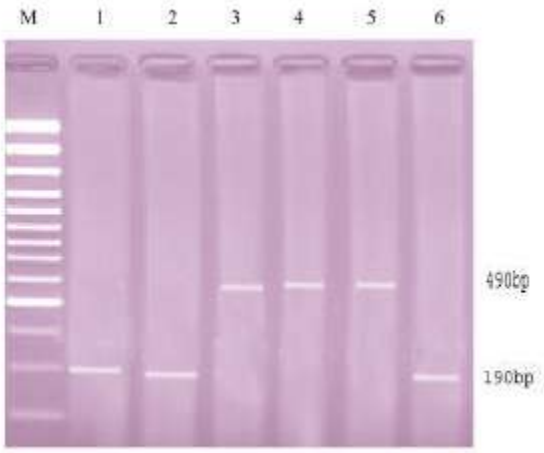

Fig. 1: Agarose gel electrophoresis stained with ethidium bromide, showing the initial amplification for ACE I/D polymorphism. Lane M represents the 100 bp ladder. The II genotype for I allele was identified by the presence of single 490 bp product (Lanes 3, 4 and 5). The DD genotype for D allele was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

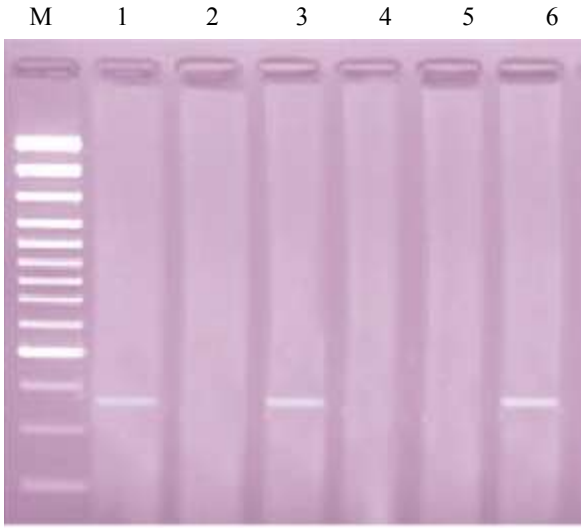

Fig. 2: Agarose gel electrophoresis of PCR products, using insertion specific primer pair, of individuals labeled as DD homozygotes following initial amplification. Absence of a product in the lanes 2, 4 and 7 confirms the presence of DD genotype. Heterozygous individuals (ID genotypes) were confirmed by the presence of a single 275 bp product (Lanes 1, 3 and6 8). Lane M represents the 100 bp.

An increase level of B2MG as well as ACE indicates the kidney failure. But to know the actual mechanism further studies have been made. Genetic predisposition studies suggest a potential role of genetic factors in the pathogenesis of renal failures at any cause 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 [27]. Hence present work was designed to study the role of ACE polymorphism of the selected community (Shettiyar) people.

The DNA samples from 100 KD, NDD nephropathy and 60 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 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 (Figure 2).

Table 3 shows the distribution of ACE genotypes in selected community based diabetic nephropathy patients and normal controls. The frequency of D allele and DD genotype was only marginally higher in both KD and

Table 3: Distribution of the genotype and allele frequencies in the study groups for the angiotensin converting enzyme (ACE) I/D polymorphism

		Genotype frequencies (percentage)			Allele frequency	
Population (n)		DD	ID	II	D allele	I allele
Control (n=60)		10 (16.6%)	33 (55.0%)	17 (28.3%)	0.441	0.559
Selected community	KD (n=100)	16 (16.0%)	54 (54.0%)	30 (30.0%)	0.44	0.56
	NDD (n=100)	18 (18.0%)	51 (51.0%)	31 (31.0%)	0.46	0.54

÷2 based on allele frequency [degrees of freedom (df) = 1], (selected community (KD and NDD) Vs Controls) = 0.00025

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

It is evident from this table that the D allele frequency of our controls was intermediate to most reported Caucasian [28-31] and Asian [32-34] populations. However, two Caucasian [35, 36] and an Asian [32] population are reported to have comparable allele frequencies. The failure to find statistically significant differences in the distribution of ACE gene I/D genotypes and their allele frequencies between the fluoride mediated nephropathy patients and the controls suggest that this polymorphism is not a risk factor for the development of renal failure in the studied population. These observations find support in the work of Tamaki *et al*. [32] and Ergen *et al*. [34].

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