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International Journal of Current Research
Vol. 10, Issue, 06, pp.70930-70936, June, 2018

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

HIGHER PREVALENCE OF DELETION ALLELE IN ANGIOTENSIN CONVERTING ENZYME GENE AMONG TYPE 2 DIABETIC SUBJECTS WITH LOWER ESTIMATED GLOMERULAR FILTRATION RATE

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ARTICLE INFO

Article History:
Received 29th March, 2018
Received in revised form
23rd April, 2018
Accepted 24th May, 2018
Published online 30th June, 2018

Key Words:

Type 2 Diabetes Mellitus (T2DM), Chronic Kidney Disease (CKD), Angiotensin Converting Enzyme (ACE) Gene Polymorphism, India.

ABSTRACT

Introduction: Chronic Kidney Disease (CKD) is associated with a high risk of developing further severe complications such as, cardiovascular disease and eventually End Stage Renal Disease (ESRD) leading to death. The aim of the study was to determine the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism in different stages of chronic kidney disease (CKD) among subjects with type 2 diabetes (T2DM). **Methods:** A total of 226 subjects with T2DM were screened for the study and classified into two groups based on estimated Glomerular filtration rate (eGFR). Those with eGFR ≥60 ml/min/1.73 m^2 (Group 1: *n* =109, M: F, 73:36) and those with eGFR <60 ml/min/1.73 m^2 (Group 2: *n*=117 M: F, 64:53). ACE genotyping was performed using Polymerase Chain Reaction. **Results:** The genotypic and allelic frequency of group 1 was II 52(47.7%), ID 47 (43.1%), DD 10(9.2%), I 151 (69.3%), D 67 (30.7%) and group 2 was II 13 (11.1%), ID 76 (65%), DD 28(23.9%), I 102 (43.6%), D 132 (56.4%) respectively. The OR (95% CI) for ID and DD genotype between the groups were 6.4 (3.2-13.1) p<0.001 and 11.2 (4.4-28.8) p<0.001 respectively after adjusting with gender, age and Body Mass Index. The genotypic frequency was also compared between genders. The OR (95% CI) for male was ID 3.1(1.3-7.4), DD 6.1(1.7-12.4), and female was ID 5.3(1.3-4.5), DD 8.8 (3.0-10.7) **Conclusion:** The ID and DD genotype of ACE gene confers a greater role in genetic variations underlying high risk stage of CKD especially in women subjects.

Citation: Vijay Viswanathan, Ezhilarasi Krishnamoorthy and Satyavani Kumpatla. **2018.** "Higher prevalence of Deletion allele in angiotensin converting enzyme gene among Type 2 diabetic subjects with lower estimated glomerular filtration rate", *International Journal of Current Research*, 10, (06), 70930-70936.

INTRODUCTION

The prevalence of chronic kidney disease (CKD) is approximately 10% in several countries (Coresh, 2007; Imai *et al*., 2009; Zhang *et al*., 2012). In India too, there is a significant burden of CKD although exact figures vary (Rajapurkar, 2012). CKD patients have high risk for cardiovascular disease and death (Go, 2004). Genetic factors, including ethnicity (Li, 2004) and family history of disease play a key role in CKD pathogenesis (Tsai *et al*., 2010; McClellan *et al*., 2012). Thus, it is desirable to identify candidate genes and evaluate their effects.

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DOI: https://doi.org/10.24941/ijcr.31189.06.2018

Angiotensin converting enzyme (ACE) is recognized as one of the main effector of renin angiotensin system (RAS) which regulates blood pressure and electrolyte balance (Yang, 1971). Owing to the key role in RAS, ACE polymorphisms have frequently been investigated. The ACE gene is localized on the long arm of chromosome 17 (17q23) and contains 26 exons. The gene is known to contain polymorphisms consisting of an insertion (I)/deletion (D) polymorphism of a 287-bp Alu-repeat sequence in intron 16 of the ACE gene that was identified and for which three different genotypes (II, ID, DD) have been characterized and used as a common marker in the susceptibility of various disorders (Mizuiri, 2001). Studies have demonstrated that ACE I/D polymorphism is associated with elevated glucose (Irvin, 2010), plasma triglyceride, total cholesterol levels, abdominal fat accumulation, central obesity and weight gain (Strazzullo *et al*., 2003) coronary artery disease (Nicholls, 1998), hypertension (Ji *et al*., 2010) and nephropathy (Amorim *et al*., 2013).

Most studies have found that carriers of the D allele had a higher risk of CKD or end-stage renal disease (ESRD) than those of I allele in the study subjects (Qin *et al*., 2012; Wang, 2012; Zhou, 2012). Our previous study also showed a strong association of D allele (ID and DD genotype) of the ACE gene polymorphism and diabetic nephropathy in T2DM subjects (Vijay *et al*., 2001). A meta-analysis also showed that CKD risk was higher in D allele carriers than in I allele carriers in Asian population (Lin *et al*., 2014). In this context, exploration of polymorphism in the ACE gene will bestow an extensive awareness about the progression of CKD and pave way for enhanced treatment options to prevent further severity of the disease (Anbazhagan *et al*., 2009). Hence, this work was aimed to study the distribution of I/D polymorphism of ACE gene in South Indian patients with low to high risk stages of CKD.

MATERIALS AND METHODS

This was a cross-sectional study, and subjects with T2DM who were attending a tertiary care centre for diabetes in South India were screened and the process of recruitment of study subjects are shown in the flow chart (Figure 1). A pilot study was first carried out using 50 subjects per group. Based on the preliminary results, with a confidence interval of 95% an estimated p value < 0.05, and a power of 80%, the present sample size were derived (Computed Using G*Power Software Version 3.1)A total of 767 subjects (M: F/438:329) were screened between February 2016 to May 2017. At the end of the study period a total of 226 subjects were included in the study. A total of 541 subjects were excluded due to the following exclusion criteria a) intake of angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) in the last 6 months (n= 373), b) T1DM (n=17), c) age > 65 yrs (n=15), d) DM duration <5 years (n=77) and e) diabetic foot ulcer (n=59). CKD is defined as kidney damage with GFR <60 ml/min/1.73m^2 for more than 3 months based on KDOQI (The Kidney Disease Outcomes Quality Initiative classification) and for this two stages were considered stages III-V (<15-59 ml/min/1.73 m^2) stages I-II (> 60 ml/min/1.73 m^2) (National Kidney Foundation, 2002). Hypertension was defined as >140 mmHg systolic blood pressure and >90 mmHg diastolic blood pressure or the use of antihypertensive therapy (Chobanian *et al*., 2003). Blood pressure was measured on the right arm with an automated blood pressure monitor while the subject was seated and allowed resting for at least 10 minutes. The study was approved by the Institutional Ethics Committee of Prof M. Viswanathan Diabetes Research Centre, Chennai, India. Informed consent was obtained from all the participants.

Anthropometric and biochemical investigations: Age, sex, height, weight and blood pressure were recorded. Body mass index (BMI) was calculated. Venous blood sample (whole blood, plasma, serum) was collected for biochemical parameters and genetic study. Biochemical analyses were done on BS 400 auto-analyzer using Diasys kits. Fasting plasma glucose was measured by GOD-POD method, serum cholesterol by CHOD-PAP method, serum triglycerides by GPO-PAP method, and high-density lipoprotein cholesterol by direct method using polyethylene glycol-pretreated enzymes. Low-density lipoprotein cholesterol was estimated directly by homogenous method. Glycosylated hemoglobin (HbA1c) was estimated by high performance liquid chromatography using the Turbo Variant machine (Bio-Rad, Hercules, CA). Serum creatinine was measured using the Jaffe method. Serum Cystatin C was measured by particle enhanced immunoturbidimetric test using DiaSys Cystatin C FS kit. Urinary albumin excretion (UAE) was measured by immunoturbidimetric method.

Isolation of genomic DNA from peripheral blood and PCR amplification: Genomic DNA was isolated from peripheral blood (Anticoagulated EDTA-blood stored at -20^{0}C) using standard phenol–chloroform extraction method with minor modification (Maniatis *et al.,* 1982). The isolated DNA was subjected to PCR amplification using T100 Thermal cycler (Bio-Rad, USA). Genomic DNA (~50 ng) was incubated in a total reaction volume of 20µl containing equal concentration of the forward primer 5′-CTG GAG ACC ACT CCC ATC CTT TCT-3′; and reverse primer 5′-GAT GTG GCC ATC ACA TTC GTC AGA T-3′ (Vijayan *et al*., 2014), 200 µM deoxynucleotide triphosphate, 10X PCR buffer pH 8.3 containing Magnesium chloride ($MgCl_2$) 15 mM and 1.5 units of Taq DNA polymerase (Genetbio) and the PCR was carried with initial incubation of 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 58°C for 30s, and extension at 72°C for 1 min, followed by a final incubation at 72 °C for 10 min. After completion of PCR cycles the product was analyzed in 2.0 %agarose gel (Sigma-Aldrich, Bangalore, India) and was captured in a gel documentation system (Bio-Rad, USA). In ACE DD genotype, a single band of 190 bp and in ACE II genotype, a band of 490 bp was observed. Both bands were observed in the heterozygous condition, 190-bp and 490-bp for ID (Figure 2). To avoid mistyping of heterozygotes (ID) DNA samples identified as a DD genotype were subsequently amplified with second set of primers designed for the insertion specific allele (Shanmugam, 1993).

Statistical analyses: All statistical analyses were performed using IBM SPSS 20.0 version software with license (SPSS Inc., IL). Mean and standard deviation for continuous variables and proportions for categorical variables are reported. Student t- test for continuous variables, Chi-square and fisher's exact test to compare categorical variables were used. In case the distribution is skewed, non parametric Mann-Whitney u test were used. A P value of <0.05 was considered to be statistically significant. The relationships between genotypes and eGFR were determined by using Univariate and multivariate logistic regression after adjusting for confounding variables.

RESULTS

Table 1 show the anthropometric and biochemical details of the study groups. There were no significant differences between the groups with respect to gender, systolic and diastolic blood pressure, fasting and postprandial glucose, HbA1c, lipid profile parameters like total cholesterol, triglycerides, HDL, LDL, VLDL and non HDL cholesterols, uric acid, duration of diabetes, duration of hypertension, history of diabetic retinopathy, neuropathy and intake of hypertension medications. However, statistically significant differences were observed in age, BMI, urea, serum creatinine, albumin/creatinine ratio, hemoglobin and diabetic medication. Differences in parameters reflecting renal function (serum creatinine, cystatin C, eGFR and urine albumin/ creatinine ratio) confirmed chronic kidney disease in group 2. Cystatin C was a better marker for the assessment of renal function than eGFR (MDRD equation mL/ min.). Cystatin C was significantly higher in group 2 (p <0.001) than in group 1.

Table 1. The anthropometric and biochemical details of the study groups

Parameter	Group I	Group II	P-Value
	eGFR ≥ 60 n=109	eGFR < 60 n=117	
Gender M:F	73:36	64:53	0.06
Age (Years)	51.6±10.5	60.3±7.9	<0.001
Body mass index (Kg/m^2)	26.3±4.2	28.5±6.6	<0.001
Systolic BP* (mm Hg)	130.4±16.5	129.5±17.2	0.71
Diastolic BP* (mm Hg)	77.6±9.5	77.8±8.8	0.82
Urea (mg/dL)	23.5±6.7	48.9±30.5	<0.001
Creatinine (mg/dL)	0.9±0.8	1.7±0.9	<0.001
*A/C ratio	10.9±11.4	19.7±28.2	0.02
*P/C ratio	-	2.2±2.7	
Serum Cystatin C (mg/L)	1.6±1.1	2.0±1.1	<0.001
Fasting Plasma glucose(mg/dL)	164.4±73.0	165.6±80.7	0.91
Post prandial glucose (mg/dL)	248.5±94.9	234.9±93.0	0.31
Glycated Hemoglobin (HbA1c) (%)	8.8±1.9	8.6±1.9	0.40
Total Cholesterol (mg/dL)	155.3±46.6	158.8±55.3	0.62
Triglycerides (mg/dL)	138.2±71.0	147.3±96.9	0.44
*HDL- Cholesterol (mg/dL)	40.0±13.2	37.7±10.8	0.17
*LDL- Cholesterol (mg/dL)	87.5±30.5	87.5±29.1	0.99
*VLDL- Cholesterol (mg/dL)	30.6±16.7	31.0±15.0	0.85
Non-HDL Cholesterol (mg/dL)	116.7±42.0	119.1±41.2	0.67
Uric acid (mg/dL)	5.3±1.9	5.3±2.3	0.93
Haemoglobin (%)	12.5±2.0	11.9±2.0	0.05
Duration of T2DM(yrs)	13.2±6.9	12.4±5.9	0.39
Duration of HTN (yrs)	2.8±2.0	3.0±2.0	0.72
Hypertention (%)	42.4	51	0.17
Neuropathy (%)	21.7	21.6	0.98
Retinopathy (%)	33.6	33.3	0.60
Diabetic medications (%)			
*OHA	33.9	18.8	
Insulin	5.5	13.7	0.01
OHA + insulin	60.6	67.5	
Hypertension medications (%)			
Alpha blockers	10	2.9	
Beta blockers	41.7	40.6	0.24
Calcium channel blockers	30	42	
Diuretics	18.3	15.5	

*BP –blood pressure, HDL- High density lipoprotein, LDL- Low density lipoprotein, VLDL- Very low density lipoprotein, HTN- Hypertension, OHA- oral hypoglycemic agent, A/c ratio- Albumin/creatinine ratio, P/C ratio- protein/creatinine ratio. Values are given in Mean± SD. Count and percentages were given for categorical variables, mean±SD were given for statistical variables.

Table 2. Shows the Univariate and multivariate logistic regression for ACE genotype in the study groups

			UNIVARIATE LOGISTIC REGRESSION				MULTIVARIATE LOGISTIC REGRESSION			
Parameter	Group -1 eGFR≥ 60 n (%)	Group -2 eGFR<60 n (%)	Unadjusted OR	95 % CI *		P-Value	Adjusted OR*	95 % CI		P-Value
II Genotype	52(48)	13(11)	Reference				Reference			
ID Genotype	47(43)	76(65)	9.64	4.1	22.5	<0.001	6.4	3.1	13.1	<0.001
DD Genotype	10(9)	28(24)	13.97	4.7	41.5	<0.001	11.2	4.3	28.7	0.001

*CI – confidential interval, *OR – ODDS ratio

Table 3. Shows the genotypic distribution of ACE gene among the male in the study groups

			UNIVARIATE LOGISTIC REGRESSION				MULTIVARIATE LOGISTIC REGRESSION			
Parameter	Group -1 eGFR≥ 60 n (%)	Group -2 eGFR<60 n (%)	Unadjusted OR	95 % CI		P-Value	Adjusted OR	95 % CI		P-Value
II Genotype	30(41)	10(16)	Reference				Reference			
ID Genotype	36(49)	38(59)	3.8	1.4	10.2	0.008	3.1	1.3	7.4	0.006
DD Genotype	7(10)	16(25)	6.8	2.1	22.0	0.005	6.1	1.7	12.4	0.001

*CI- Confidential interval, *OR- ODDS ratio

Table 4. Shows the genotypic distribution of ACE gene among the female in the study groups

			UNIVARIATE LOGISTIC REGRESSION				MULTIVARIATE LOGISTIC REGRESSION			
Parameter	Group -1 eGFR≥ 60 n (%)	Group -2 eGFR<60 n (%)	Unadjusted OR	95 % CI		P-Value	Adjusted OR	95 % CI		P-Value
II Genotype	22(61)	3(6)	Reference				Reference			
ID Genotype	11(31)	38(72)	6.9	1.7	10.7	<0.001	5.3	1.3	4.5	<0.001
DD Genotype	3(8)	12(22)	9.3	5.1	16.4	<0.001	8.8	3.0	10.7	<0.001

*CI – confidential interval, *OR – ODDS ratio

Table 5. Allelic frequencies of I/D polymorphism of ACE gene in study subjects

Parameters	eGFR ≥ 60 n=109	eGFR < 60 n=117	OR (95 % CI)	P-Value
	n (%)	n (%)		
I allele				
Overall	151(69)	102(44)	0.3(0.2 - 0.5)	<0.001
Male	96(66)	58(45)	0.4(0.26-0.7)	0.001
Female	55(76)	44(42)	0.2(0.1-0.4)	<0.001
D allele				
Overall	67(31)	132(56)	2.9(1.9-4.2)	<0.001
Male	50(34)	70(55)	2.3(1.4-3.8)	0.001
Female	17(24)	62(58)	4.6(2.4-8.9)	<0.001

*CI – confidential interval, *OR – ODDS ratio

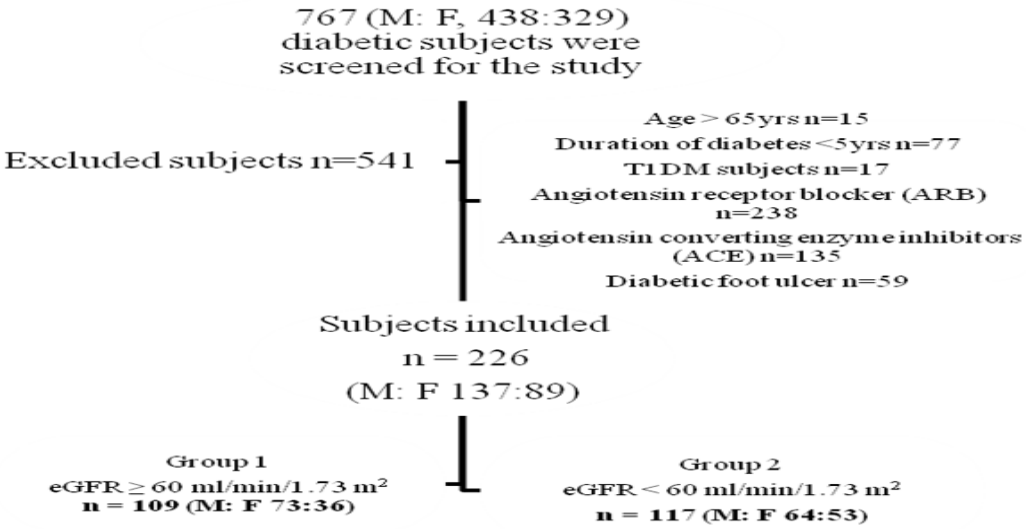

Figure 1: Flow chart showing screening and recruitment of the study subjects.

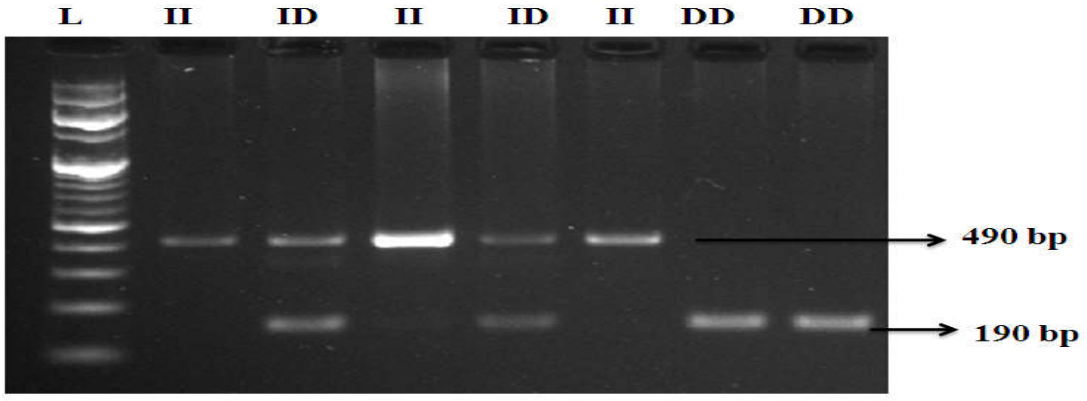

Figure 2. Representative band patterns of ACE I/D polymorphism analyzed using agarose gel electrophoresis Lane L represents 100bp ladder, II- 490bp, ID- 490 & 190bp, DD- 190bp

The genotypic and allelic frequency of groups as follows II 52(47.7), ID 47 (43.1), DD 10(9.2), I 151 (69.3), D 67 (30.7) for group 1and II 13 (11.1), ID 76 (65), DD 28(23.9), I 102 (43.6), D 132 (56.4) for group 2. The OR (95% CI) for ID and DD genotype between the groups were 6.4 (3.2-13.1) p<0.001 and 11.2 (4.4-28.8) p<0.001. Multivariate logistic regression analysis was done to evaluate whether ACE polymorphism was associated after adjusting for age, gender and BMI (Table 1). The ID and DD genotype was significantly prevalent in group 2 after adjusting with gender, age and BMI. The genotypic frequency was also compared between gender. The OR (95% CI) for male was ID 3.1(1.3-7.4), DD 6.1(1.7-12.4), and female was ID 5.3(1.3-4.5), DD 8.8 (3.0-10.7). The risk was 6.4 times higher in group 2 after adjusting for confounding factors for ID genotype. The gender wise frequency of II, ID, DD genotypes was shown in table 3 and 4. The Univariate analysis showed that male subjects are at 3.8 times risk for ID genotype and 6.8 times risk for DD genotype (Table 3). Whereas female subjects were at 6.9 times risk for ID genotype and 9.3 times risk for DD genotype (Table 4). After adjusting with confounding factors the risk remains the same with slight variations. The overall, male and female allelic frequency of I and D allele was shown in table 5. The overall risk for I allele was significantly low with not much difference between male and female subjects. Whereas for D allele overall odds ratio risk was 2.9. Female subjects (OR=4.6) are twice the risk than male subjects (OR=2.3, p<0.001).

DISCUSSION

Genetic variations can be used as a marker to identify the individuals at risk. Many association studies have been carried out to identify genetic risk factors that predispose to various diseases. Our previous study showed a strong association of D allele (ID and DD genotype) of the ACE gene polymorphism and diabetic nephropathy in T2DM subjects (Vijay et al., 2001) with small sample size. In the present study we analyzed the distribution of ACE genotype in risk stages of CKD among T2DM subjects. Our present cross sectional study showed significant association of ID and DD genotype with high risk stage of CKD. Only few studies have been done in CKD subjects of south Indian population.

Two of the studies showed no significant association in the genotypic and allelic frequencies between CKD cases and controls. The genotypic and allelic frequency were II 27(23.0), ID 58 (49.0), DD 33 (28.0), I 112 (47.5), D124 (52.5). Glomerular filtration rate (GFR) was estimated by using Modification of Diet in Renal Disease (MDRD) equation (Kolandaswamy *et al*., 2009). Another study with a total of 147 CKD patients included was under CKD stages (II–V) as diagnosed and identified by the nephrologists. The eGFR estimated method was not mentioned. The genotypic and allelic frequency were II 45(30.61), ID 62 (42.17), DD 40 (27.21), I 152 (52.0), D 142 (48.0). This study also showed no significant association when compared to control subjects. The DD genotype was found to be the major risk determinant of CKD among female (OR = 2.4) population (Nagamani *et al*., 2015). In our present study also the major risk was found to be associated with D allele in female subjects with risk of 4.6 times. However, our results are contradictory to above two studies done by Kolandaswamy *et al*., and Nagamani *et al.,* and similar to one of the studies which showed significant difference in CKD subjects by Shanmuganathan *et al.,* (Kolandaswamy *et al*., 2006; Nagamani *et al*., 2015; Shanmuganathan *et al*., 2015) . The study manifests strong association of ID and DD genotype of ACE gene polymorphisms and CKD. The study consists of 60 CKD stages III-V (<15-59 ml/min/1.73 m2) subjects and the genotypic and allelic frequency were II, 4 (6.7%), ID, 30 (50%) and DD, 26 (43.3%), I, 38(31.6%), D, 82(68.4%) (Shanmuganathan *et al*., 2015). The genotypic and allelic frequency was similar to the present study which supports our findings. CKD mainly affects older population (>50 years) (Riordan, 2005). A significant difference in age was noted between the groups in our study. The subjects with CKD were older. Obesity is one of the strongest risk factors for new-onset CKD (Elsayed, 2008; Tsujimoto *et al*., 2014). The BMI was significantly increased in group 2 than group 1 subjects and this shows increase of BMI is also one of the strongest factors for CKD progression.

It was found that D allele, ID and DD genotype was associated with female subjects in our study. Similar observations were also reported in different populations. In 2010, Mansoor *et al*., have reported that the ACE I/D polymorphism was associated with female population (OR (CI) = 1.95 (0.99–3.85) and p value 0.05) rather than male population (OR (CI) =0.81 (0.52–1.26) and p value 0.35) (Mansoor *et al*., 2010). Another study by Pinon *et al*. found that the DD genotype is the risk factor for the development of renal disease (Pinon *et al*., 2009). Further, a meta-analysis from Lin *et al*. stated that the gender dependent effect of the ACE I/D polymorphism have been observed very commonly among different populations (Lin *et al*., 2014). Thus gender wise analysis showed that D allele, ID and DD genotype found to be the major risk determinants of CKD among female (OR = 4.6, 5.3 and 8.8) population. This result was also supported by previous reports from France (Hadjadj *et al*., 2003) and Mexican population (Dhanachandra Singh *et al*., 2014). Furthermore, recent study from South India reported that female subjects are more prone to hypertension after menopause in Tamilnadu. The study showed that the DD genotype is associated with the female population (OR-CI = 2.40 (1.05–5.51),p = 0.04) as compared to the male population (OR-CI = 0.75 (0.37–1.51), p = 0.42). The general consensus is that as kidney function declines, there is a proportional increase in Hypothalamic–pituitary–gonadal (HPG) axis dysfunction, ultimately leading to loss of menses, infertility, and functional menopause.

Although women with CKD may have comorbidities and characteristics that may independently affect ovarian function, such as diabetes, the presence of kidney disease itself also appears to play a role (Mnterrosa- Castro et al., 2013). However kidney disease may adversely affect HPG axis function is unclear, but there are a number of hormonal alterations. The cyclic release of gonadotropin-releasing hormone is reduced in CKD, leading to the loss of pulsatility in luteinizing hormone (LH) and follicle-stimulating hormone (FSH), release and ultimately decreased estradiol secretion and anovulation. Hyperprolactinemia in CKD is common and is the result of both increased pituitary production due to a resistance to the inhibitory effects of dopamine as well as decreased renal excretion (Hou, 1985). Increased levels of prolactin results in a decrease in normal cyclic gonadotropin-releasing hormone secretion, which ultimately results in the loss of pulsatile release of LH and FSH, thereby leading to a decrease in estrogen release, the clinical implications being irregular menses, infertility, and functional menopause.

In the present study, females in group 2 are above 60 years probably post-menopausal women. In group 1, 11 females and in group II, 33 females are above 60 years of age. In group 1, 13 females and in group II, 28 females are with post-menopausal state. Based on these observations we hypothesized, the DD genotype is associated with increased activity of the ACE and subsequent CKD among female population (Nagamani *et al*., 2015). Fava *et al*., have reported that the DD genotype of the ACE polymorphism is associated with a more rapid decline in eGFR and with an increased mortality in type 2 diabetic patients with established renal disease. The study also showed that ACE genotyping might be useful in selecting patients with poorer prognosis and may results in more aggressive treatment (Fava et al., 2001). The discrepancy in results in different population might be due to genetic heterogeneity, environmental background or different study designs and methods.

Limitations

The present study has provided only a genetic association for I/D polymorphism of the ACE gene among South Indian patients when those with lower and higher eGFR were compared and not compared with controls. We included one tertiary care center in our study; More CKD patients from different kidney centers can be included for better interpretation for the role of ACE I/D polymorphism or the progression of CKD. However, we failed to analyze the ACE levels in the study subjects. More studies can be conducted using cohort and RCT study design with larger sample size and control group.

Conclusions

In conclusion, the study highlights that ID and DD genotype (D allele) of ACE gene confers a greater role in genetic variations underlying CKD. These results suggest that CKD patients with moderate risk should be offered analysis for variation in ACE I/D polymorphisms especially in women of South Indian population, and these can also be used as a genetic marker for susceptibility to nephropathy in this population.

Acknowledgements

We acknowledge the help rendered by Mr. Sriram in statistical analysis. We would like to thank Ms. Bliss for her help in sample collection. We also thank all the study subjects who contributed to this research.

Source of Support: Prof. M. Viswanathan Diabetes Research Centre.

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