Synonymous Variant of *ACE* Gene (*rs4343*) is Coupled with Early Age at Onset and Diminished Diabetic Duration in South Indian Diabetic Nephropathy Patients

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ABSTRACT

Diabetic nephropathy (T2DN) is a major micro vascular complication associated with type-2 diabetes (T2DM). Abnormally active Renin Angiotensin System pathway is shown to influence the development of T2DN and the inter-individual variation with respect to *ACE* levels, a key enzyme in this pathway partly dependent on the genetic variants of *ACE*; suggests patient's genotype may influence the progression and severity of diabetes and its complications. The current study explores the impact of synonymous variant of *ACE rs4343* in the propensity, age at onset and disease duration of T2DM and T2DN in south Indians. This case-control study includes blood samples from 100 T2DN, 100 T2DM and 100 healthy controls (HC). Genomic DNA was extracted, PCR-RFLP based genotyping of *ACE rs4343* polymorphism was performed and appropriate statistical analysis was done. Elevated frequency of G allele and GG genotype in T2DN group in comparison with T2DM patients and HC was noted ($p<0.05$). Among the T2DN vs. T2DM patients with ≤ 45 yrs of age GG genotype showed 3.4 fold increased risk towards development of nephropathy ($p<0.05$). Further, GG imparted a 3 fold elevated risk

among T2DN patients having ≤ 7 years of diabetic duration before the onset of nephropathy than the patient group with ≥ 7 years of diabetic duration ($p<0.05$). Multiple Logistic Regression analysis revealed BMI, W/H ratio, AAOM, DODM, and GG genotype as an independent risk factors for the development of T2DN. To our knowledge this is the first study of *rs4343* polymorphism in the perspective of age at onset and progression of T2DN presenting a significant association of mutant genotype with both the aspects in south Indian population. To realize these findings studies with large sample are warranted in various ethnic group.

Keywords: *ACE rs4343* polymorphism; Diabetic Nephropathy; Age at onset and Disease duration of diabetes.

INTRODUCTION

Discerning the biological role of key bio-molecules play an important role in understanding the pathophysiology of numerous human diseases including type-2 diabetes (T2DM) which pave the path for their better management. As biochemical reactions are mediated by enzymes, variations in their genes may pose an advantage or disadvantage to the individuals carrying them towards complex diseases. One such gene Angiotensin Converting Enzyme (*ACE*) that was extensively studied in relation to variety of diseases impacted heavily in the management of several *ACE* dependent diseases (Sayed-Tabatabaei FA et al., 2006). Diabetic nephropathy (T2DN) a major micro vascular complication of T2DM is the largest single cause of end stage renal failure and abnormally active Renin-Angiotensin System (RAS) is shown to influence the development of T2DN. The inter-individual variation with respect to *ACE* levels, a crucial enzyme that converts angiotensin I to angiotensin II in RAS pathway partly reliant on the genetic variants of *ACE*, signifying its genotype may influence the progression and severity of diabetes and its complications (Chawla T et al., 2010). A large number of functional polymorphisms exist in this gene, *rs4343* a synonymous mutation (Thr776Thr) in exon 17th is reported to alter mRNA folding that decrease its stability and transcription level (Keavney B et al., 1998; McKenzie CA et al., 2005). The HAPMAP revealed a varying frequency of minor allele of *rs4343*polymorphism in different populations, the least being in the African (0.3) and the highest in European population (0.54). The mutant minor allele is associated with increased serum *ACE* levels suggesting, high levels of *ACE* may independently or in interaction with other genes or environment may exert an effect that impact the genetic predisposition of an individual to the disease or course of the disease differently in different populations (Zhu X et al., 2001).

The present study sought to investigate the role of *rs4343* polymorphism in the propensity, age at onset of diabetes and diabetic duration before the onset of nephropathy in south Indians. The importance of this study lies in the event of reduction in the age at onset of diabetes and diminishing duration of diabetes before the onset of T2DN globally as well as in our population.

MATERIALS AND METHODS

Sampling and data collection

This case-control study comprises a total of 300 subjects of southern India. Patients diagnosed by an endocrinologist and nephrologists, of whom 100 were clinically identified type-2 diabetic nephropathy group (T2DN),100 type 2 diabetic patients without nephropathy (T2DM) and 100 healthy controls (HC) visiting Nizam's institute of Medical sciences hospital (NIMS), Hyderabad, India. The present study was approved by Ethical committee of Maulana Azad National Urdu University, Hyderabad, India. Demographic, anthropometric details like age, gender, height, weight, waist and hip measurements, family history of the disease and duration of diabetes were recorded in a well-designed Performa. Informed consent was obtained prior to the sample collection; procedure and motive of the study were explained to the patients. Body mass index (BMI) and waist to hip ratio (W/H) was calculated as given elsewhere (Snehalatha C et al., 2003). Blood samples were obtained from the subjects for

biochemical and molecular analysis. In order to see the influence of *rs4343* polymorphism on the age at onset of diabetes (AAODM) patients (T2DM+T2DN) were categorized into two age groups i.e. ≤ 45 years and > 45 years. Further stratification of DN patients were done based on duration of diabetes before onset of nephropathy into two groups 1) patients who develop nephropathy within 7 years (≤ 7) of diabetes 2) those who develop nephropathy after 7 years (>7) of diabetes duration.

Inclusion criteria

The inclusion criteria for the three different study groups were: a) T2DN patients: Individuals diagnosed with a urine albumin excretion of >300 mg/day on two different occasions in a span of 3-6 months without any clinical evidence of other kidney diseases and infectious condition. b) T2DM individuals without nephropathy: urinary albumin excretion of <30 mg/day (checked on three different occasions) and negative for dipstick urinary protein, longstanding diabetes for a period of 10 years with no history of hypertension prior to the development of diabetes. c) HC: unrelated healthy individuals with no history of diabetes, hypertension and renal disorders.

Biochemical analysis

Readings of Fasting and Post lunch Blood sugars (FBS, PLBS) were obtained from the medical records of the hospitals. Glycosylated hemoglobin (HbA1c) levels were estimated using a Nycocard reader (AXIS-SHIELD, Norway). The serum creatinine, albumin levels and lipid profile were measured by enzymatic methods in a semi-auto analyzer.

Molecular analysis

Genomic DNA was isolated from venous blood by using salting out method. The quantity and quality of DNA was assessed with spectrophotometer and gel electrophoresis. The genotyping for the *ACE rs4343* (A2350G in exon 17) polymorphism was performed using PCR-Restriction Fragment Length Polymorphism (RFLP). The amplification reaction was carried out in a total volume of 10µl, using 100ng genomic DNA, 1µl of buffer, 0.3µl of dNTP, 0.2µl of each forward 5'AAGGAGAGGAGAGAGACTCA3' and reverse primer 5'TGTTGGCAGCAGGGACTCACC3' (Bioserve Biotechnologies, Hyderabad, India) 0.3µl of Taq polymerase (Labpro, Hyderabad, India). The PCR cycling conditions were: an initial denaturation at 95°C for 5 min followed by 30 cycles, including denaturation at 94°C for 30 sec, annealing at 58o C for 45 sec, and a final extension at 72°C for 5 min. The restriction enzyme BtsCI (Labpro, India) was used to distinguish the *rs4343* genotyping. The digested PCR product showed the fragment size of 432 bp for mutant type (G) and 290 bp and 141 bp for wild type (A) On 2% agarose gel electrophoresis (Figure 1). Negative and positive controls were used in each genotyping run and few samples were randomly picked and re-genotyped to avoid mistyping of genotypes.

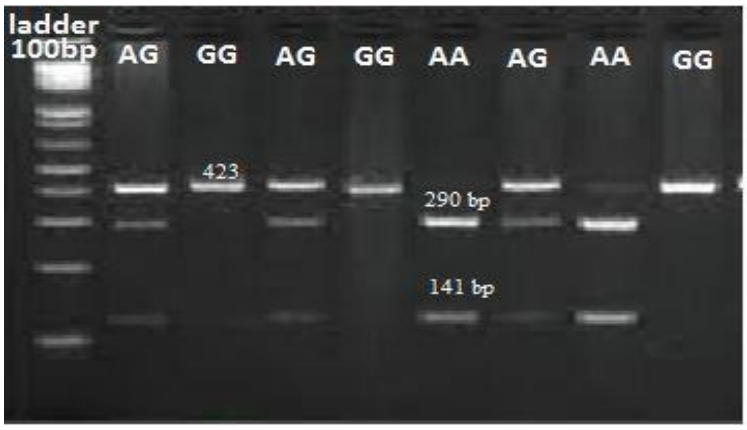

Figure 1: Representative gel picture of *ACErs4343* polymorphism

Statistical analysis

Descriptive statistics was done to calculate percentages, mean and standard deviation (SD). Student's t-test was used to test for differences in various characteristics for continuous variables. The evaluation of data among the groups was performed by ANOVA (one-way ANOVA) test. Genotypic and allelic frequencies among study participants were analyzed with the Chi-square test and Hardy Weinberg Equilibrium (HWE) was calculated. Chi-square contingency tables were used to compare the allele and genotype frequencies between patients, controls and subgroups. The risk associated with genotypes was estimated using online odds ratio calculator at 95% confidence interval (CI). Relationship between the risk factors and T2DN was assessed through Multiple Logistic Regression (MLR) analysis. Data analysis was carried out by SPSS version 21 wherever required.

RESULTS

Characteristics of the study group

A total of 300 subjects were recruited for the present study. The baseline anthropometric and clinical features of the study population are summarized in Table 1. Results are expressed as mean ± SD in healthy controls (HC), T2DM and T2DN. Two-way ANOVA was performed with respect to various anthropometric and clinical features to compare the means between HC, T2DM and T2DN groups and their mean ages at the time of sample collection was 62.67 ± 5.6, 59.37 ± 7.86 and 60.6 ± 7 years respectively.

Table 1: Demographic and clinical characteristics of the study group.

	HC (n=100) X±SD	**T2D (n=100) X±SD**	**T2DN (n=100) X±SD**	***p*-value**
Age (years)	62.67 ± 5.6	59.37 ± 7.86	60.6 ± 7	**0.003**
Males (%)	50 (50)	50 (50)	50 (50)	-
Females (%)	50 (50)	50 (50)	50 (50)	-
Mean of #AAODM	-	49.5 ± 6.1	45.91 ± 5.53	< **0.0001***
BMI (Kg/m^2)	23.7 ± 3.51	24.95 ± 2.96	23.58 ± 3.4	**0.006**
W/H ratio	0.83 ± 0.08	0.93 ± 0.06	0.91 ± 0.03	<**0.001**
HbA1c (%)	4.9 ± 0.6	8.15 ± 1.62	8.76 ± 1.51	<**0.001**
Serum creatinine (mg/dl)	0.82 ± 0.19	1.14 ± 0.54	3.96 ± 2.38	<**0.001**
Serum albumin (g/dl)	4.2 ± 0.55	4.07 ± 0.61	3.03 ± 0.67	<**0.001**
Total cholesterol (mg/dl)	152.12 ± 31.21	178.72 ± 42.77	202.61 ± 49.97	<**0.001**
High density lipoprotein (mg/dl)	48.77 ± 5.34	42.68 ± 11.96	30.83 ± 11.09	<**0.001**
Low density lipoprotein (md/dl)	71.44 ± 17.64	109.58 ± 46.33	140.03 ± 55.77	<**0.001**
Very low density lipoprotein	17.85 ± 6.43	28.94 ± 17.99	32.02 ± 9.44	<**0.001**
Triglycerides (mg/dl)	114.47 ± 34.72	171.1 ± 86.71	196.65 ± 61.33	<**0.001**
TC/HDL ratio	3.26 ± 0.75	4. ± 0.7	7.55 ± 3.41	<**0.001**

One way anova analysis for all the variables (except mean of AAODM) was performed as a test of significance; *t-test p-value; #AAODM= age at onset of diabetes mellitus.

Genotype association analysis

Table 2 represents the distribution of *ACE rs4343* genotypes, allelic frequencies and their odds ratio among the studied population. The current analysis revealed that percentage distribution of GG genotypes was more in T2DN (39%) when compared to T2DM (20%) and HC (18%) subjects. The frequency of AG genotype was marginally high among T2DM (43%) than T2DN (39%) and HC (33%). Higher frequency of AA genotype was found among HC (49%) than T2DM (37%) and T2DN (22%) individuals. The G and A allele frequencies were 0.35, 0.65; 0.42, 0.58 and 0.59, 0.42 respectively in HC, T2DM and T2DN subjects. The *ACE rs4343* genotype frequencies were found to be in Hardy Weinberg Equilibrium (HWE) among the T2DM group ($\chi2 = 1.31$, $p = 0.25$) whereas, a deviation of the genotype frequencies from HWE was observed in the HC and T2DN groups ($\chi2 = 7.28$, $p = 0.006$; $\chi2 = 3.87$, $p = 0.05$ respectively). Overall analysis between patients and control group (T2DN+T2DM vs. HC) revealed that GG genotype to be imparting 2 fold increased risk toward diabetes (OR-1.91; 95% CI=1.05–3.45; $p<0.05$) whereas AA genotype was found to be protective (OR-0.43 95% CI=0.26–0.71; $p <0.001$). The subgroup analysis between HC and T2DM did not show any genotype dependence ($p>0.05$), while between HC and T2DN as well as between T2DM and T2DN groups replicated the results as that of overall patients and controls with varying folds and significance levels ($p <0.05$).

Table 2: Distribution of genotype and allele frequency of ACE rs4343 gene polymorphism among patients and controls.

Category (N)	AA (%)	AG (%)	GG (%)	χ2 value (p-value)	Allele frequency		Group Comparison	OR (95% CI)	*p*-value
					G	A			
Patients (DM+T2DN) (200)	59(29.5)	82(41.0)	59(29.5)	11.59 (0.003)	0.50	0.50	AA vs. AG + AA AG vs. AA + GG GG vs. AA + AG	0.43 (0.26-0.71) 1.41 (0.85-2.33) 1.91 (1.05-3.45)	**0.001** 0.2 **0.03**
HC (100)	49(49)	33(33)	18(18)		0.35	0.65			
HC (100)	49(49)	33(33)	18(18)	3.09 (0.21)	0.35	0.65	AA vs. AG + AA AG vs. AA + GG	0.61 (0.34 - 1.07) 1.53 (0.86 - 2.72)	0.11 0.18
DM(100)	37(37)	43(43)	20(20)		0.42	0.58	GG vs. AA + AG	0.13 (0.56 - 0.71)	0.85
HC (100)	49 (49)	33(33)	18(18)	18.50 (<0.01)	0.35	0.65	AA vs. AG + AA AG vs. AA + GG	0.33 (0.17 - 0.61) 1.29 (0.72 - 2.31)	**0.0003** 0.46
T2DN (100)	22 (22)	39 (39)	39 (39)		0.59	0.42	GG vs. AA + AG	2.91 (1.52 - 5.57)	**0.001**
DM(100)	37(37)	43(43)	20(20)	10.13 (<0.01)	0.42	0.58	AA vs. AG + AA AG vs. AA + GG	0.53 (0.28 – 1.0) 0.85 (0.48-1.59)	0.06 0.66
T2DN (100)	22(22)	39(39)	39(39)		0.59	0.42	GG vs. AA + AG	2.55 (1.35-4.81)	**0.005**

H.W.E:HC-$\chi2$= 7.28 (p 0.006), **T2DM Patients**-$\chi2$=1.31 p:0.25, **T2DN Patients** –$\chi2$= 3.87, p:0.05

Figure 2 represent the *ACE rs4343* genotype distribution in relation to AAODM among T2DM and T2DN patients. In ≤ 45 years category, both G allele and GG genotype were found to be elevated in T2DN and T2DM groups (0.68% vs. 0.46%; 51.78% vs. 23.91%). While, an enhanced frequency of A allele and AA genotype were observed in T2DM group compared to T2DN group (0.54% vs. 0.32%; 32.61%vs. 16.07%). No genotype variation was observed among the older age group i.e. >45 years ($p>0.05$).

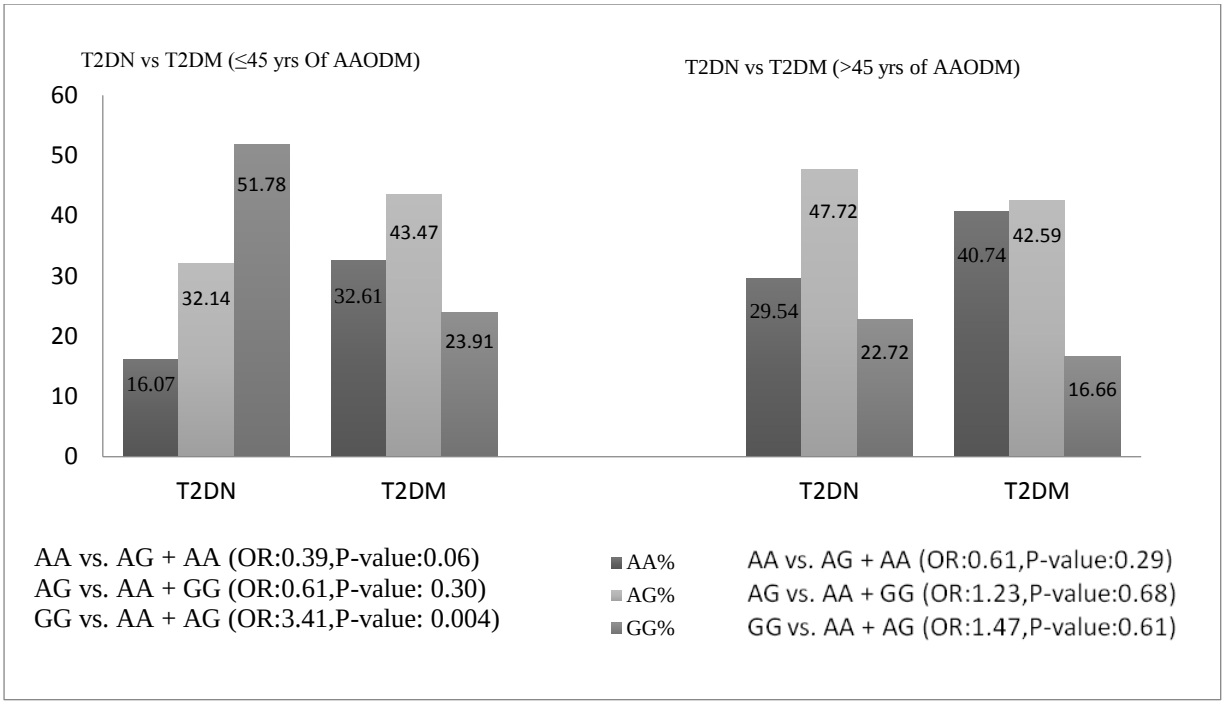

Figure 2: Genotype distribution of ACE rs4343 polymorphism based on AAODM among T2DM and T2DN patients.

Figure 3 shows the genotype distribution of T2DN patients based on the DODM prior to the onset of nephropathy. There was a 3 fold increased risk for patients with GG genotype to develop nephropathy within ≤ 7 years of DODM and AA genotype seemed to have a protective effect, as patients with this genotype were represented more in the latter group.

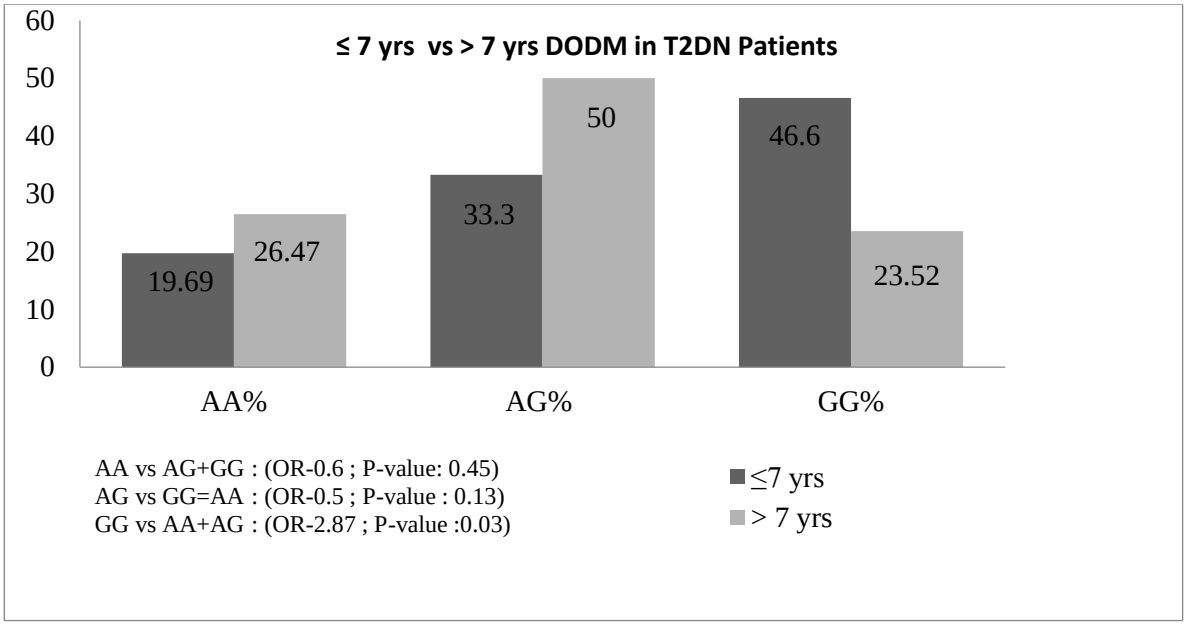

Figure 3: Genotype distribution of ACE rs4343 polymorphism among T2DN patients based on the DODM.

Multiple Linear regression analysis was done aiming at identifying the independent risk factors for T2DN. Among T2DN vs. T2DM groups MLR revealed that BMI, W/H ratio, AAODM, DODM and *ACE rs4343* GG genotype were associated with an enhanced risk for T2DN (Table 3).

Table 3: Multiple Logistic Regression Analysis results of ACE rs4343 in T2DNvs. T2DM group.

Variables	OR (95% CI)	*p*-value
Gender	0.75 (0.35-1.6)	0.46
BMI	1.65 (1.01-2.69)	**0.05***
W/H Ratio	1.68 (0.45-3.76)	**0.04***
AAODM	1.89 (0.86-1.99)	**0.02***
DODM	1.11(1.08-1.24)	**0.01***
HbA1c	2.09 (1.29 -5.33)	0.99
T.C.	1.85 (0.68-5.02)	0.22
TC/HDL ratio	2.56 (0.35-18.47)	0.11
T.G.	1.19 (0.51-2.76)	0.67
GG genotype	2.43 (1.08-5.47)	**0.03***

*$p<0.05$

DISCUSSION

Asian Indians, an ethnically distinct population, lead the world in the number of people with type-2 diabetes (T2DM) and associated complication nephropathy (T2DN) which contributes both in terms of morbidity, mortality and socioeconomic loss due to End stage renal failure. T2DM is a complex disorder whose etiopathophysiology involves numerous genetic and environmental factors and their interactions (Wild S et al., 2004; De Fronzo RA et al., 2015; Lin YC et al., 2018). The efforts of scientists in understanding the implicated minor and major genes both in the genetic predisposition and progression of T2DM and its complications will aid to the better management of these conditions.

Based on the experimental and epidemiological data it is well established that activation of the RAS pathway plays an important role in elevating the micro and macro vascular problems in patients with T2DM (Gurley SB, Coffman TM 2007). *ACE* inhibitors have been reported to improve kidney, heart function, vascular compliance, endothelial derived nitric oxide production, vascular relaxation, plasma markers of inflammation, oxidative stress and thrombosis inT2DN individuals. The favorable effects of *ACE* inhibitors are due to the influence on hemodynamic as well as tissular effects of Angiotensin II (Cordonnier DJ et al., 2001; Samy I McFarlane et al., 2003).

Angiotensin converting enzyme is one of the crucial enzyme in RAS pathway. The inter-individual variation seen among the subjects with respect to *ACE* levels is dependent on the gene variants of *ACE*, suggesting its genotype may influence the severity and progression ofT2DM and T2DN (Rahimi Z et al., 2012). A large number of polymorphisms exist in *ACE* gene with functional significance. *rs4343* polymorphism is one among them, a synonymous variant that is associated with increased *ACE* levels. Generally the synonymous variants alter the gene expression either through change of codon usage frequency, a change in mRNA secondary structure and stability or a change in normal splicing pattern (Chamary JV et al., 2006; Joanna L Parmley and Hurst 2007).

McKenzie et al., 2005 demonstrated G allele of *rs4343* polymorphism leads to significant change in *ACE* expression by altering mRNA folding (McKenzie et al., 2005).

This genetic variant of *ACE* gene has not been sufficiently explored as well there are no studies pertaining to south Indian population (Table 4). Inflammation and oxidative stress (OS) coupled disorders are shown to be associated with elevated levels of Angiotensin II and diabetes is one among them with chronic low grade inflammation and increased OS. The primary objective of the current study was of three fold, to see whether this polymorphism has an impact on (i) the propensity of T2DM and T2DN. (ii) the age at onset ofT2DM and T2DN. (iii)The duration of diabetes (DODM) before the onset of nephropathy.

Table 4: rs4343 studies in different populations on related to Diabetes / Nephropathy.

Authors	Year	Country / Population	Disease
Parul Aggarwal	2016	N.India	Gestational diabetes
P.huo, D, et al	2015	N.Chinese	Diabetic Nephropathy
Cheema et al	2013	Asian Indians	Type 2 Diabetic
Ahluwalia TS, et.al.	2009	Asian Indian	Type 2 diabetic nephropathy.
Narita, et.al.	2003	Japanese	IgA nephropathy

In the present study significant augmented association of mutant G allele and GG genotype in T2DN, A allele and AA genotype in T2DM individuals suggest their predisposing and protective roles towards nephropathy. Our results are in conformity with Huo P et al. (2015) who demonstrated a link between GG genotype of *rs4343* polymorphism with T2DN compared to that of T2DM patients indicating that G allele imparts susceptibility towards nephropathy (Huo P et al., 2015). Su SL et al. (2012) reported an association of this polymorphism with chronic kidney disease in Chinese Han population (Su SL et al., 2012). Contrary to above reports, Ahluwalia et al. (2009) showed lack of association in North Indian diabetic nephropathy patients (Ahluwalia TS et al., 2009). At large this advocates *ACE* may be an important factor in contributing to kidney diseases through Ang II mediated vasoconstriction, inflammation, OS and fibrosis. Auxiliary investigations with regard to this variant of *ACE* gene in diverse populations are necessary, given the limitation of the present study by the sample size.

The second objective of the current study was to see the influence of *rs4343* on the AAODM among the patient group. Generally early-onset diabetes is considered to be with an age cutoff of 30–45 years. Even though globally T2DM is considered as a disease allied to aging, the prevalence of adult early-onset type has increased and higher proportion of early-onset T2DM was reported in Asian countries with a 4-fold increase from 1997 to 2010 in China than in Western countries (BK Lee et al., 2016). In our study we observed that 40% of T2DM patients developed the disease with in ≤ 45 years (45 years mean age at onset) and 60% of patients after 45 years of age (52 years mean age at onset. Further, we found that Diabetic patients with ≤ 45 years of age and carrying two mutant alleles exhibited a threefold increased tendency towards nephropathy than the T2DM patients with other genotypes, suggesting the possible interaction of this genotype with lifestyle factors. Further studies dealing with lifestyle factors may help in understanding the modifying effect of this polymorphism on the age at onset of T2DM and T2DN.

The third objective of ours was to investigate the influence of *rs4343* polymorphism on the DODM before the onset of nephropathy within the T2DN group. The observation of increased frequency of patients with the mutant homozygous genotype who developed T2DN ≤ 7 years of diabetic duration compared to those who developed nephropathy after 7 years of T2DM suggest that the high producing genotype may speed up the progression or reduce the time of progressing towards nephropathy. It is well known that overt nephropathy caused by glomerulosclerosis appears after 5 to 10 years in patients with T2DM and the chances of nephropathy increases

with the duration of T2DM (Inassi J and Vijayalakshmy 2013). Based on our results we conclude that *rs4343* polymorphism has a role not only in the susceptibility but also in AAO and DODM of T2DM and T2DN.

T2DM and T2DN being a complex condition depends on multiple intrinsic and extrinsic factors. We sought to explore the independent effect of potential risk factors for T2DN using MLR which has reemphasized the role of the GG genotype, AAO and DODM in the occurrence of nephropathy. In addition BMI and W/H ratio also emerged out as contributing factors (Schuler R et al., 2017). A large study considering various anthropometric and lifestyle factors is warranted to substantiate these observations.

CONCLUSION

In conclusion, we found an association of *ACE rs4343* polymorphism with both T2DM and T2DN in our population. Further we suggest that this polymorphism influences not only the genetic predisposition to diabetes and diabetic kidney disease, but also the age at onset and duration of disease before the setting in of the complications. The limitation of the study is small sample size and lack of information of *ACE* levels. Large studies considering age at onset, duration of disease and various lifestyle factors among different ethnic groups substantiate the results and will be valuable in the management or therapeutic intervention through *ACE* inhibitors.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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