ORIGINAL ARTICLE

Distribution of Angiotensin Converting Enzyme I/D Polymorphism in Diabetic and Diabetic Nephropathy Patients at a Tertiary Care Hospital in Maharashtra

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Abstract:

Background: Diabetes Mellitus (DM) is one of the leading non-communicable disorders, leading to various complications viz. cardiovascular diseases, retinopathy, nephropathy, neuropathy and peripheral vascular disorders. Diabetic Nephropathy (DN) patients further develop into End Stage Renal Disease (ESRD) and they have to undergo the repeated blood transfusions, increasing the social and economic burden. The number of risk factors are known for causation of diabetic nephropathy including the environmental, biochemical as well as genetic factors. The association of nephropathy with various genes has been proved. *Aim and Objectives:* In the present study we attempted to check the association of Insertion/Deletion (I/D) polymorphism of Angiotensin Converting Enzyme (ACE) in diabetic patients with and without nephropathy and also with the biochemical markers. *Material and Methods:* Each group consisted of 110 individuals viz. diabetics with and without nephropathy and age and gender matched healthy controls. *Results:* The determination of I/D polymorphism by polymerase chain reaction revealed the significant increased 'D' allele frequencies in patients of diabetes with and without nephropathy than the controls, while no significant difference was noted in genotype frequencies. The odds ratios for this polymorphism were calculated to be 1.84 and 2.41 for DM and DN respectively in comparison with the healthy controls. The regression analysis indicated I/D polymorphism is associated positively with all the lipid parameters, except High Density Lipoprotein-Cholesterol (HDL-C) which was negatively associated with the polymorphism. The levels of lipid parameters were also significantly increased in patients of diabetes with and without nephropathy carriers for 'D' allele than the patients having 'I' allele, while the level of HDL-C was significantly decreased. *Conclusion:* The conclusion can be made from these results that, the presence of I/D polymorphism of ACE may increase the risk of development of nephropathy in general population, with the role of 'D' allele in its causation, along with its effect on the biochemical markers.

Keywords: Diabetic nephropathy, Diabetes mellitus, Angiotensin converting enzyme, I/D polymorphism, lipid markers

Introduction:

The incidence of metabolic syndrome, Diabetes Mellitus (DM) and obesity is created by combination of genetic and environmental factors increasing dramatically worldwide [1]. With the largest number of DM patients, India has earned a dubious distinction of being the "Diabetes capital of the world" [2-3]. After the earliest documentation of DM, it was recorded almost 3000

years ago, is known to affect the humans [4]. The disorder DM is characterized by chronic hyperglycemia with multi etiological origin and the metabolisms of carbohydrate, fat and proteins are known to be affected due to defects in insulin secretion and/or action [5].

In 2003, the dramatic increase in DM population was observed worldwide, and it is likely to increase more rapidly in future. About 5.1% of the world population i.e. around 194 million people were suffered with DM globally. The projected number of DM patients by the year 2025 is around 330 million worldwide, and will be endemic in Asia. The projected number of DM patients with higher incidence of Type 2 DM (T2DM), is 73 million in India over next 25 years from 36 million [6-8].

As the hyperglycemia develops gradually and it is not severe enough to produce the classical symptoms in early stages of the disease, T2DM patients remain undiagnosed for many years, due to which these patients are at higher risk of developing micro and macro vascular complications with high morbidity and mortality. The manifestations include the metabolic syndrome, a combination of Cardiovascular Diseases (CVD) risk factors, which apart from glucose intolerance include dyslipidemia, hypertension, visceral obesity, hypercoagulability and microalbuminuria. T2DM patients shortly after the diagnosis, show the presence of either microalbuminuria or overt nephropathy [9].

Diabetic nephropathy was the entity unknown till the invention of insulin therapy, but increase in the horizon was seen after World War II, which increased the patients of End Stage Renal Disease (ESRD) with diabetes [10]. About 20-30% of DM patients develop nephropathy, which is independent of cause of hyperglycemia and is the most common single cause associated with a high incidence of ESRD [5, 11].

The expenditure on the T2DMpatients undergoing dialysis is considerably higher than the ESRD patients without diabetes [10]. In the U.S., Diabetic Nephropathy (DN) accounts for about 40% of new cases of ESRD, and 18 to 30 billion dollars of yearly costs may be utilized by it in coming decade [4, 9]. It is estimated that in India about 1, 00,000 people suffer from ESRD annually, of which only about 20,000 get treated [11]. DM patients with Chronic Kidney Disease (CKD) prior to development of ESRD expend more time in hospital than patients without any complications. Almost one third population of India is poverty-stricken, though the cost of the dialysis in India is inexpensive compared to other countries, but almost 90%of Indian population cannot bear the expenses [12].

To diagnose the patients with higher renal risk in T2DM patients, clinically a positive family history is very useful [13]. Strong evidence suggests that genetic inheritance in families of both type 1 and type 2 diabetics has very important role in development of DN [14, 15]. The alterations in Renin Angiotensin System (RAS) may lead to the hemodynamic changes [15].

The important role may be played by RAS in the pathogenesis of DN. Highly polymorphic genes have been noted in the genes of RAS in number of genetic studies, and have been suggested as potential risk factors in combination with environmental factors. Therefore, the RAS gene make up is altered and the functioning of RAS is affected in individuals. One of the important enzymes of RAS, Angiotensin Converting Enzyme (ACE) gene is 21 kb long DNA sequence situated on chromosome 17 with 26 exons. Within

intron 16, the insertion or the deletion of 287 bp fragment characterizes the Insertion/Deletion (I/D) polymorphism in ACE gene, which has been studied considerably with renal [9] and CVD [16] complications of DN. 'D' allele is related with elevated ACE activity, and thereby nephropathy [14, 15]. Therefore, we hypothesized that the pathogenesis of DN may be progressive with presence of ACE DD allele and it may be associated with CVD markers.

Material and Methods:

The present case-control study was carried out in the Department of Biochemistry, Bharati Vidyapeeth (Deemed to be University) [BV (DTU)] Medical College and Hospital and Diabetes Laboratory, Interactive Research School for Health Affairs, Pune, Maharashtra. The study protocol was approved by Institutional Ethics Committee (BVDU/MC/55/2013-2014), BV (DTU) Medical College, Pune. A total of 220 clinically diagnosed patients of T2DM were selected from Department of Medicine, BV (DTU) Medical College and Hospital, Pune. Among 220 T2DM patients, 110 were T2DM patients without nephropathy, and 110 were T2DM patients with nephropathy. T2DM with nephropathy patients again were sub-divided into two groups viz.

i. T2DM patients with microalbuminuria (n=55) (Urine albumin level between 30 to 300 mg/24 hours)

ii. T2DM patients with proteinuria (n=55) (Urine albumin level more than 300 mg/24 hours). One hundred ten were age and gender matched healthy individuals as controls; the controls were selected without any evidence of DM, CKD or chronic liver diseases, and other endocrine diseases. Informed consent was obtained from all participants.

Collection and Storage of Samples:

After getting informed consent from individual participants included in the study, patients were advised for overnight fasting for about 12-14 hours. Venous blood samples from antecubital vein were collected with aseptic precautions. Total of 6 ml blood was collected. The samples were collected in plain vacutainers, two hours after collection, the samples were centrifuged at 3000 rpm for 15 minutes, clear and un-hemolysed sera were collected in polythene tubes. The tubes were corked, labeled and stored at -80°C until analysis for Total Cholesterol (TC), Triglycerides (TG), High Density Lipoprotein-Cholesterol (HDL-C), urea and creatinine. The levels of Low Density Lipoprotein-Cholesterol (LDL-C) and Very Low Density Lipoprotein-Cholesterol (VLDL-C) were calculated by Friedewald's formula [17]. Fasting Plasma Glucose (FBG) was estimated from fluoride vacutainer. The EDTA blood samples were used for the determination of HbA1c, and isolation of DNA for mutational analysis. The commercially available kits from Randox laboratories Ltd., United Kingdom were used for estimation of biochemical parameters.

Genetic Analysis of ACE I/D:

The DNAs from all participants were extracted from whole blood by salting out method [18] and analyzed for the presence or absence of polymorphism I/D of ACE by Polymerase Chain Reaction (PCR) [19].

The primers used for I/D polymorphism of ACE gene were forward primer 5'-GCCCTG CAGGTGTCTGCAGCATGT-3' and reverse primer 5'-GGATGGCTCTCCCCGCCTTG TCTC-3'. The primers were synthesized commercially (Eurofins Genomics, Germany).

The PCR was performed in total volume of 25µl. After vortexing briefly, PCR Master Mix (Qiagen) was dispensed 12.5 µl into each PCR tube (2.5 units of TaqDNA Polymerase, 2x QIAGEN PCR Buffer, 3 mMMgCl2, and 400 µM of each dNTP), followed by addition of 0.5 µl of each primer, 1 µl DNA template and final volume was made to 25 µl with sterile distilled water. The PCR tubes were kept on ice until they were placed in the thermal cycler. The PCR was done with initial denaturation of 10 min at 94°C, followed by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 40 seconds at 58°C, and extension for 30 seconds at 72°C, and 1 cycle of final extension for 7 min at 72°C. The amplified PCR products were resolved by agarose gel electrophoresis on 2% agarose gel (Low EEO, Bangalore Genei, India). The image was captured under a UV-transilluminator Gel Doc™XR+ (Bio-Rad, United States). In the case of homozygous for I/I and D/D genotypes, one band of 597 bp and 319 bp was observed respectively. And both bands are visible in heterozygotes for I/D polymorphism (Fig. 1).

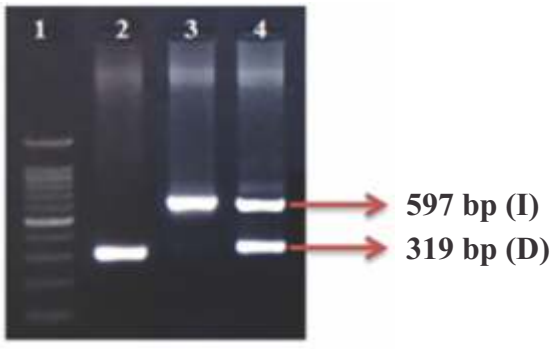

Fig. 1: Genotyping of ACE I/D Polymorphisms by PCR
Lane 1: 100 bp DNA Ladder
Lane 2: Homozygous for 'D' allele
Lane 3: Homozygous for 'I' allele
Lane 4: Heterozygous for I/D allele

Statistical Analysis:

The frequencies of Single Nucleotide Polymorphisms (SNPs) were calculated and compared statistically by Chi-square test between T2DM patients with and without nephropathy and controls. The odds ratios were calculated for each group with respective 95% Confidence Interval (CI). Using logistic regression analysis, the association of ACE I/D polymorphism and biochemical markers was tested. The quantitative parameters were calculated as Mean ± Standard Error (SE) and the effect of I/D polymorphism on demographic and biochemical variables within subgroups (II Vs. ID+DD) of ACE gene was compared using students unpaired't' test. The results were considered significant when $p<0.05$. Statistical analysis was done using SPSS for Windows, version 21 and SNP Stat online software.

Results:

Detection of ACE gene I/D Polymorphism:

The genomic DNAs were isolated from blood samples of all subjects. The DNA concentration and purity was confirmed using the relative absorbance ratio at 260nm/280nm. DNA samples with a 260nm/280nm ratio higher than 1.8 were used for ACE I/D polymorphism detection by PCR. The frequencies of genotypes of ACE I/D polymorphism in patients of T2DM, with and without nephropathy and controls were calculated. In T2DM patients without nephropathy, 28 (25.5%) subjects were homozygous for the 'I' allele (II), 25 (22.7%) subjects were heterozygous i.e. ID and 57 (51.8%) subjects were homozygous for D allele (DD). In T2DM patients with nephropathy, 18 (16.37%) subjects were homozygous for the 'I' allele (II), 33 (30%) subjects were heterozygous i.e. ID and 59 (53.63%) subjects were homozygous for D allele (DD). In control group, 43 (39%) subjects

were homozygous for the 'I' allele (II), 28(25.5%) subjects were heterozygous i.e. ID and 39 (35.5%) subjects were homozygous for the D allele (DD) for ACE I/D polymorphism. The 'I' allele frequencies were 0.31, 0.37 and 0.52 and the minor allele 'D' frequencies were 0.69, 0.63 and 0.48 in T2DM patients with and without nephropathy and controls, respectively. Frequencies of genotypes and alleles of ACE I/D polymorphism in study population and controls are shown in Table 1. The odds ratios with 95% CI and significance level are shown in table 2.The odds ratios were calculated for ACE I/D polymorphism. The odds ratio for ACE I/D in DN patients against DM patients was found to be 1.30, but it was not significant, therefore no effect of this polymorphism was noted in development of DN in DM patients. In contrast to this the odds ratios for ACE I/D in DM and DN patients against controls were found to be 1.84 and 2.41 with significant p value of 0.0335 and 0.0028 respectively. Therefore, general population has 1.84 times higher risk of DM and 2.41 times higher risk of DN if the individual is carrying the ACE D/D allele.

Table 1: Frequencies of Genotypes and Alleles of ACE Gene I/D Polymorphism in T2DM without Nephropathy, T2DM with Nephropathy and Controls

Genotypes	Controls (n=110)	DM (n=110)	DN (n=110)	DN vs. DM	DM vs. Controls	DN vs. Controls
Genotype frequencies n (%)						
II	43 (39)	28 (25.5)	18 (16.37)	0.6222	0.7140	0.1122
ID	28 (25.5)	25 (22.7)	33 (30)			
DD	39 (35.5)	57 (51.8)	59 (53.63)			
Allele frequencies						
I	0.52	0.37	0.31	0.4555	0.0464	0. 0041
D	0.48	0.63	0.69			

**statistically significant if p⩽0.05, DM: T2DM patients without nephropathy; DN: T2DM patients with nephropathy*

Table 2: Odds Ratio and 95% Confidence Interval in ACE gene I/D Polymorphism between Groups

	DN vs. DM	DM vs. Controls	DN vs. Controls
Odds ratio	1.3072	1.84	2.41
95% CI	0.7269 to 2.3508	1.0489 to 3.244	1.3535 to 4.2959
z statistic	0.895	2.126	2.987
P	0.3709	0.0335	0.0028

**statistically significant if p⩽0.05, DM: T2DM patients without nephropathy; DN: T2DM patients with nephropathy*

Association of ACE I/D Polymorphism with Biochemical Parameters:

Logistic regression analysis between ACE I/D polymorphism and cardiovascular markers showed positive association of ACE I/D polymorphism with BMI (p=0.0071), TC (p=0.0004), LDL-C (p<0.0001), TG (p=0.0086) and VLDL-C (p=0.0086), and negative association of this variant was found with HDL-C (p<0.0001). Logistic regression analysis between ACE I/D polymorphism and cardiovascular markers showed positive association of ACE I/D polymorphism with TC (p<0.0001), LDL-C (p<0.0001), TG (p<0.0001) and VLDL-C (p<0.0001), and negative association with HDL-C (p<0.0001) within T2DM subjects without nephropathy. Body Mass Index (BMI), FBG, HbA1c, blood urea, serum creatinine, urine creatinine, urine microalbumin and Albumin to Creatinine Ratio (ACR) did not exhibit significant association with this polymorphism in T2DM patients with nephropathy. The statistical findings of logistic regression analysis i.e. regression coefficient, standard error, and p values are shown in Table 3.

Table 3: Logistic Regression Analysis of ACE I/D with Biochemical Parameters in Patients of T2DM, with and without Nephropathy

Variables	Regression Coefficient	Standard Error (SE)	*P*
With nephropathy			
BMI (kg/m^2)	0.33869	0.12579	0.0071
Cholesterol (mg/dL)	0.22542	0.063796	0.0004
HDL-C (mg/dL)	-0.22168	0.049433	<0.0001
LDL-C (mg/dL)	0.13839	0.033126	<0.0001
Triglycerides (mg/dL)	0.300682	0.11684	0.0086
VLDL-C (mg/dL)	1.53826	0.58521	0.0086
Without nephropathy			
Cholesterol (mg/dL)	0.17	0.038584	<0.0001
HDL-C (mg/dL)	-0.54402	0.10441	<0.0001
LDL-C (mg/dL)	0.17916	0.041375	<0.0001
Triglycerides (mg/dL)	0.10072	0.020481	<0.0001
VLDL-C (mg/dL)	0.50307	0.10236	<0.0001

**statistically significant if p⩽0.05, BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; VLDL-C: Very low density lipoprotein cholesterol*

Effect of ACE I/D Polymorphism on Biochemical Parameters in Diabetic Nephropathy Patients:

To study the effect of ACE I/D polymorphism genotypes on biochemical parameters; we compared biochemical parameters among different I/D genotypes for this variant. The comparison of biochemical parameters was done on the basis of presence or absence of 'D' allele of ACE I/D polymorphism in T2DM patients with and without nephropathy. It was found significantly higher BMI (p=0.0046), TC (p<0.0001), TG (p<0.0001), LDL-C (p<0.0001) and VLDL-C (<0.0001), and decreased HDL-C (p<0.0001) within T2DM subjects with nephropathy carrying 'D' allele (I/D+D/D) as compared to those with homozygous for 'I' allele among ACE I/D polymorphism. It was observed that FBG, HbA1c, blood urea, serum creatinine, urine creatinine, urine microalbumin and ACR did not differ statistically among these genotypes in T2DM patients with nephropathy. The mean values of biochemical and lipid parameters with Standard Error (mean ± SE) are depicted in Table 4 between homozygotes for 'I' allele and carriers for 'D' allele (ID+DD) for ACE I/D polymorphism in T2DM patients with nephropathy.

Table 4: Comparison of Biochemical and Lipid Parameters within Subgroups of ACE I/D Polymorphism in T2DM Patients with Nephropathy

Parameters	ACE I/I (N=18)	ACE (I/D+D/D) (N=92)	*P*
BMI (kg/m^2)	26.19 (0.7)	28.1 (0.26)	0.0046
FBG (mg/dL)	159.26 (9.98)	190.96 (8.56)	0.11
HbA1c (%)	8.83 (0.62)	8.86 (0.28)	0.97
Blood urea (mg/dL)	65.71 (9.82)	76.23 (5.05)	0.39
Serum creatinine (mg/dL)	1.33 (0.11)	1.47 (0.09)	0.41
Urine creatinine (g/L)	5.33 (0.57)	5.23 (0.22)	0.86
Urine microalbumin (mg/L)	296.11 (40.1)	301.34 (17.49)	0.9
A/C Ratio (mg/g)	53.83 (4.05)	55.38 (1.88)	0.74
Cholesterol (mg/dL)	147.6 (3.45)	215.11 (4.34)	<0.0001
Triglycerides (mg/dL)	99.87 (5.76)	210.94 (8.08)	<0.0001
HDL-C (mg/dL)	49.53 (1.58)	37.12 (0.97)	<0.0001
LDL-C (mg/dL)	78.09 (4.12)	135.81 (3.87)	<0.0001
VLDL-C (mg/dL)	19.97 (1.15)	42.19 (1.62)	<0.0001

**statistically significant if p⩽0.05, BMI: Body mass index; FBG: Fasting blood glucose; A/C ratio: Albumin/creatinine ratio; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; VLDL-C: Very low density lipoprotein cholesterol*

Effect of ACE I/D Polymorphism on Biochemical Parameters in T2DMwithout Nephropathy:

A significant increase in the levels of TC ($p<0.0001$), TG ($p<0.0001$), LDL-C ($p<0.0001$) and VLDL-C ($p<0.0001$), while decrese in HDL-C ($p<0.0001$) level was observed in T2DM patients without nephropathy having D allele (I/D+D/D) as compared to the T2DM homozygous patients with 'I' allele for ACE I/D polymorphism.BMI, FBG, HBA1c, blood urea, serum creatinine, urine creatinine, urine microalbumin and ACR did not differ statistically. Mean ± SE of biochemical and lipid parameters are depicted in Table 5 between patients carriers for 'D' allele (I/D+D/D) and homozygotes for 'I' allele of ACE I/D polymorphism in T2DM without nephropathy.

Table 5: Comparison of Biochemical and Lipid Markers within Subgroups of ACE I/D Polymorphism in Patients of T2DM without Nephropathy

Parameters	ACE I/I (N=28)	ACE (I/D+D/D) (N=82)	*P*
BMI (kg/m^2)	27.47 (0.53)	26.79 (0.28)	0.24
FBG (mg/dL)	133.93 (3.32)	130.76 (1.71)	0.37
HbA1c (%)	7.51 (0.3)	8 (0.15)	0.12
Blood urea (mg/dL)	39.87 (1.59)	37.65 (0.87)	0.21
Serum creatinine (mg/dL)	1.61 (0.08)	1.5 (0.05)	0.29
Urine creatinine (g/L)	1.25 (0.06)	1.2 (0.03)	0.5
Urine microalbumin (mg/L)	19.59 (0.8)	18.7 (0.39)	0.28
A/C Ratio (mg/g)	15.94 (0.34)	15.79 (0.18)	0.69
Cholesterol (mg/dL)	164.2 (2.6)	204.87 (2.43)	<0.0001
Triglycerides (mg/dL)	137.7 (2.35)	172.3 (2.38)	<0.0001
HDL-C (mg/dL)	52.02 (0.56)	41.49 (0.46)	<0.0001
LDL-C (mg/dL)	84.63 (2.75)	128.93 (2.42)	<0.0001
VLDL-C (mg/dL)	27.54 (0.47)	34.46 (0.48)	<0.0001

**statistically significant if $p \leq 0.05$, BMI: Body mass index; FBG: Fasting blood glucose; A/C ratio: Albumin/Creatinine ratio; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; VLDL-C: Very low density lipoprotein cholesterol*

Discussion:

The changes in the genetic make-up of genes can be determined and the knowledge can be utilized for the improved diagnosis of substantial cases of the disease which is a major public health burden in industrialized nations, and can also be useful in treatment or prevention of the disease. The aim to study the common allelic variants was to find out the risk of these diseases in the general population, which will be useful for the prediction of the diseases in future [20].

The factors including hypertension, diabetes duration and glycemic control alone cannot lead to pathogenesis of DN and define the high risk of DN, along with these the genetic and environmental factors play an important role. The development of DN in diabetic patients is found to be controlled genetically. Irrespective of the glycemic control of the diabetic patients, 35% of them developed DN as per the epidemiological studies [21].

Numbers of susceptible genes have been studied with their role in the predisposition of various microvascular complications [22].The genes previously analyzed in hypertension, CVD and T2DM have been also assessed in DN patients [23].Polymorphisms in various genes have been shown to be included in the pathogenesis of DN including PPAR-γ, eNOS, GLUT-1, aldose reductase, MTHFR, ApoE and components of the RAS including angiotensinogen, angiotensin II receptor type 1, and particularly, the ACE gene [24].

An exopeptidase, angiotensin-converting enzyme, is known to be important in various physiological functions [23]. It generates angiotensin II, a vasoconstrictor peptide and inactivates bradykinin and angiotensin, thereby helps in the homeostasis of blood pressure [25]. Via generation of angiotensin II, it is also responsible for microcirculation regulation in the kidney [26]. The lower concentration of ACE can limit intrarenal generation of angiotensin II, therefore increase in ACE activity increases angiotensin II in glomeruli, and thereby increases intraglomerular hydraulic pressure which favors the development of diabetic glomerulosclerosis [27].

The ACE is 21 kb gene with 26 coding exons and 25 non-coding introns and it is situated on short arm of chromosome 17.Almost 160 polymorphisms in ACE gene have been studied, most of which are SNPs. Of these all polymorphisms the first report on I/D polymorphism was in 1990 by Rigat *et al.* [28] for the first time, which indicates either presence (insertion) and absence (deletion) of a fragment of DNA sequence (287 bp) from intron 16 of ACE gene.

Since 1990, ACE I/D polymorphism association with DN has been studied exclusively, with report of more than 300 studies, of which almost 100 studies evaluated the association of DN with ACE I/D polymorphism [16]. The presence or absence of repetitive Alu sequence in intron 16 of this polymorphism, lead to three genotypes (DD and II homozygotes and ID heterozygotes) [22, 29]. Staessen *et al*. [30], in a meta-analysis reported 1.56 times higher risk of DN with presence of DD genotype against presence of II genotype.

We investigated T2DM patients with and without nephropathy for the presence or absence of I/D polymorphism ACE gene, and healthy controls. No significant genotype distribution between any of the three study groups was detected. The 'D' allele frequencies insignificantly differed among DM and DN patients, while 'D' allele frequency was significantly increased in patients of DM and DN than healthy controls. Logistic regression

analysis revealed, the presence of allele 'D' of ACE I/D polymorphism was positively associated with total cholesterol, triglycerides, LDL-C and VLDL-C, while it was negatively associated with HDL-C in T2DM patients with and without nephropathy. BMI was also positively associated with presence of 'D' allele of this polymorphism in only DN patients.

Significant increase in the levels of lipid markers viz. TC, TG, LDL-C, VLDL-C was noted, while HDL-C was decreased in T2DM patients with and without nephropathy, which were carriers for 'D' allele than the homozygotes for 'I' allele. BMI was significantly increased in the carriers for 'D' allele than 'I' homozygotes in Type 2 diabetic nephropathy patients.

One of the most important factors in the susceptibility to DN via the presence of ACE polymorphism is ethnicity. The inconsistent results were noted in various studies regarding the role of ACE I/D polymorphism in DN pathogenesis. The association of presence of 'D' allele of ACE gene with DN was found in Asian population, while there was no association between two in Caucasians [26].

Several studies have found the 'D' allele to be an independent risk factor for DN and it is used as a marker in population structure analyses. Patel *et al*. [29] in Western Indian population reported, ACE gene I/D polymorphism not found to affect the pathogenesis of DM and non-DN complications, while, association of the DD polymorphism has been reported in development of DN. Hussein *et al*. [31] highlighted significant association of dominant model with the risk of DN which rose by three folds. They also showed that the minor allele 'D' frequency was significantly higher in DN as compared to controls. In accordance with the present study, Arfa *et al*. [32] and Jayapalan *et al*. [24] showed no association of (I/D) polymorphism within the ACE gene with DN nor with DM in the Tunisian and Malaysian populations, respectively. Bhaskar *et al*. [23] reported insignificant differences in genotype frequencies of ACE I/D polymorphisms when DN patients were compared with controls, in South Indian population. Mizuiri *et al*. [33] in Japanese population tried to correlate the level of renal ACE mRNA with ACE I/D genotype in healthy individuals, and found the association of mRNA expression of ACE with ACE I/D polymorphism.

Similar results were reported from South India by Khan *et al*. [22]. No significant difference in genotype frequency distribution of ACE I/D polymorphism was detected by these authors in T2DM patients with nephropathy and controls but they observed a significant association of ACE-D allele with susceptibility to diabetic nephropathy. Viswanathan *et al*. [34] in South Indian T2DM patients also reported a positive association between 'D' allele of the ACE I/D polymorphism and proteinuria in diabetics.

Like DN I/D polymorphism of ACE gene is found to be the significant factor to increase the risk of other non-communicable disorders including CKD, coronary artery disease, Coronary Heart Disease (CHD) and hypertension. An increased risk of DN and presence of DD genotype are genetically strongly associated [29]. As the polymorphism is found in an intron, it has no effect on the structure of the enzyme [31]. The presence of ACE I/D polymorphism found to be associated with ACE levels in plasma, inside the cell, and tissue [26, 33].

Plasma levels of ACE enzyme are under genetic control. 40% of variation in ACE activity in serum and renal tissue in individuals is reported due to presence of ACE I/D polymorphism [16]. Through

this the physiological functions mediated via ACE are influenced, and also its genetic susceptibility to glomerular lesions [23, 24, 27]. Deletion (D) homozygous individuals were found to have the highest levels of plasma ACE; heterozygous individuals were having intermediate levels, while individuals homozygous for the insertion had the lowest levels of plasma ACE [26]. Therefore in homozygotes for 'D' allele with high ACE activity, the increase in level of vasoactive peptide angiotensin II is noted, which increases GFR and blood pressure, contributing to development of DN, it also induces Transforming Growth Factor-β1 (TGF-β1), and intraglomerular pressure leading to the development of DN, thereby simulates mesangial cell proliferation and production of extracellular matrix, and bradykinin inactivation released from the variety of tissues affecting arterial vasodilation and vasoconstriction [26, 29]. It also induces production of oxygen radicals, at least *in vitro*. In addition, the angiotensin II molecule stimulates the production of the mineral corticoid hormone aldosterone, which increases the blood pressure by increasing salt re-absorption [26]. Therefore, from the present study and the studies from literature it is evident that, the presence of 'D' allele of ACE I/D, play an important role in the development of diabetic nephropathy in diabetes mellitus patients by increasing the activity of ACE enzyme. The presence of 'D' allele, also shown to be associated with the lipid levels as well as BMI, therefore giving a link of dyslipidemia with pathophysiology of nephropathy in diabetic patients

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