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Role of eNOS and TGFβ1 gene polymorphisms in the development of diabetic nephropathy in type 2 diabetic patients in South Indian population

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Abstract

Background: Diabetic nephropathy is known to be a leading complication of diabetes mellitus, characterized by diverse aspects such as high urinary albumin level, elevated blood pressure, and genetic susceptibility leading to end-stage renal disease. The current study was carried out to investigate the association of *eNOS* and *TGFβ1* gene polymorphisms in the progression of diabetic nephropathy among type 2 diabetic patients in the South Indian population. The *eNOS* and *TGFβ1* genetic variants were genotyped in 280 T2DM patients, 140 with DN, 140 without DN, and 140 controls. Genotyping was performed using ARMS PCR and the genomic variants were confirmed by the Sanger sequencing method.

Results: A significant ($p < 0.05$) association was observed in the genotypic frequencies of eNOS (G > T) polymorphism in the T2DM patients with diabetic nephropathy when compared to controls. The frequency of TT (heterozygous) genotype was observed to increase in patients with type 2 diabetes and DN when compared to the diabetic patients without DN and controls. This indicates that diabetic patients with TT genotype are at an increased risk to develop DN. However, *TGFβ1* (G > C) polymorphism did not show any association in the allele and genotypic frequencies with DN when compared with T2DM and controls.

Conclusion: The results of the study propose a strong influence of TT genotype of *eNOS* gene be significantly linked with diabetic nephropathy in T2DM patients. Whereas no association was examined concerning *TGFβ1* gene polymorphism and DN. Nevertheless, large sample size studies are required to confirm the part of these genetic variants in the development of DN.

Keywords: Diabetic nephropathy, Gene polymorphism, Microalbuminuria, eNOS, TGFβ1, Type 2 diabetes mellitus

Background

Diabetic nephropathy (DN) is recognized to be a distinct and substantial microvascular problem of both type 1 (T1DM) and type 2 diabetes mellitus (T2DM) and is a prominent source of end-stage renal disease (ESRD) [1]. DN is characterized by an early stage of microalbuminuria, deteriorating glomerular filtration rate, and hypertension that over a period of time leads to a progressive phase which ultimately drives to kidney failure, further necessitating dialysis or transplantation. Hypertension is a key factor that is known to increase the risk of DN [2]. Several factors such as high blood sugar, high blood pressure, and genetic variations might influence an individual towards DN in the immediate future [3].

The endothelial nitric oxide synthase (eNOS) gene is considered to be a promising candidate gene to

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study the predisposition of diabetic nephropathy [4, 5]. This gene has three polymorphisms such as *894G > T* (rs1799983) missense mutation found in exon 7, 786 T > C (rs2070744) polymorphism found in the promoter region and 27 bp repeat (4b4a) found in the intron 4 region. All of these single nucleotide polymorphisms are found to be linked to the prognosis of diabetic nephropathy. The eNOS gene is proved to be one of the main factor behind endothelial dysfunction which is a hallmark of DN. The variants of the eNOS gene are proved to contribute to endothelial dysfunction as well as diminish the production of nitric oxide. The NOS3 regulation at the transcriptional, post-transcriptional and post-translational levels is known to be influenced by the genetic variants in the eNOS gene. The incidence of G894T variant causes decreased level of nitric oxide and is linked with hypertension as well. Also the genetic polymorphisms present in the eNOS gene is known to contribute in the development of endothelial dysfunction and thereby reduces the production of nitric oxide. [6] Among the polymorphisms, *G894T* was proved to intensify the threat of macroalbuminuria and the advancement of microalbuminuria to macroalbuminuria with a deterioration in the glomerular filtration rate as per the rise in serum creatinine levels subsequently causing diabetic nephropathy [7]. Studies have proved that the T allele, as well as TT genotype of eNOS 894G > T, polymorphism have a significant risk of developing DN. An increased prevalence of this polymorphism is observed among Japanese and Tunisian diabetic patients which further became the reason for renal failure [8].

Transforming growth factor-beta 1 (TGF-β1) is considered as an anti-inflammatory immune negotiator that obstructs or reverses the stimulation of macrophages by interrupting with signaling by using toll-like receptor-reliant pathways [7]. *TGFβ1* gene comprises seven exons and is situated at the chromosome 19q13.1 [9, 10]. Various single nucleotide polymorphisms of *TGF-β1* have been studied some among them are 869 T > C polymorphism, where the Leucine changes to Proline in the codon 10, −509 C > T was another SNP which was proved to be associated with a concentration of circulating TGF- β1 protein. Another study investigated the effect of G915C polymorphism and proved its relationship with an enhanced risk of end-stage renal disease [11]. A meta-analysis study claimed that no association was observed concerning 915G > C polymorphism and diabetic nephropathy [12].

Despite all the investigations done on these two genes and their polymorphisms in the earlier case–control studies and meta-analysis studies it's still hard to come to a conclusion about the link between these genes in the progression of diabetic nephropathy.

Aim

The aim of the present investigation is to examine the association of the genetic polymorphism of *eNOS* (rs1799983) and *TGFβ1* (1800471) in diabetic patients with and without diabetic nephropathy and their risk of developing DN among the South Indian type 2 diabetic patients.

Methods

The present investigation was a prospective case–control study performed in a tertiary care hospital. The patient samples were collected and evaluated from November 2017-December 2019. During this period 280 type 2 diabetic patients with and without DN (137 men and 143 women; mean age 56.4 ± 5.8 years) attending the outpatient department of Nephrology and General medicine of a tertiary care center were recruited. Similarly, 140 healthy participants (60 men and 80 women; mean age 55.1 ± 6.8 years) were studied as a control group. The participants were dispersed into three different groups. Group A -140 type 2 diabetes mellitus patients with diabetic nephropathy, Group B- 140 type 2 diabetes mellitus patients without diabetic nephropathy, Group C- 140 healthy subjects. The clinical and demographic characteristics were obtained from all the subjects by means of a structured questionnaire and the other methodology such as, anthropometric measures, clinical examination and laboratory investigations. It included gender, age, BMI, diabetes duration, FPG, PPG, serum creatinine, HDL, LDL, VLDL, eGFR, systolic and diastolic blood pressure, smoking, and alcohol consumption. Type 2 diabetic patients were diagnosed by means of ADA criteria 2012, where the FBS level was > 126 mg/dl and PPG level was > 200 mg/dl [13]. Diabetic nephropathy patients were assessed by the level of urinary protein-to-creatinine excretion of > 30 mg/g in a 24-h urine collection. The patients with DN were diagnosed based on the KDOQI (Kidney Disease Outcomes Quality Initiative) guidelines [14]. The participants for the study were strictly selected based on the clinical examination.

Selection criteria

The participants included for the study were male and female participants with diagnosed type 2 diabetes mellitus, subjects with age between 28 and 70 years, urinary protein-to-creatinine ratio > 30 mg/g in 24 h urine sample collection, albumin creatinine ratio (ACR) level between 30–299 mg/g and ≥ 300 mg/g in spot urine collection and patients with decreased eGFR levels. The participants excluded from the study were patients with

proteinuria before the onset of diabetes, other complexities such as heart disease, urinary tract infection, and with inadequate records were left out from the study group, participants having any infectious disease and drug-induced group as well as subjects with other kidney problems. The healthy control participants were normal healthy participants in the age limit of 28–70 years old, with no history of diabetes mellitus or any other diseases.

DNA isolation

The blood samples were collected in ethylene-diamine tetra acetic acid (EDTA) anticoagulant vacutainer tubes and were stored at −4°C for further isolation as well as genotyping of DNA. 5 ml of whole blood was collected from the control and case subjects. The DNA extraction was done by using Miller's method [15]. The quality and quantity of DNA were observed with a spectrophotometer followed by agarose gel electrophoresis.

Primer design and T-ARMS-PCR amplification

The primers designed to target the two SNPs were performed by using an online software established by Ye et al., and accessible at http://primer1.soton.ac.uk/primer1.html. Tetra primer-amplification refractory mutation system-PCR (T-ARMS PCR) is known to be a rapid, effective, and cost-efficient technique for the identification of mutations or SNPs [16].

eNOS G894T (rs1799983) genotyping

The PCR product sizes for the variant rs1799983 polymorphism were as: 277 bp for two outer primers (control bands), 175 bp for the G allele, and 158 bp for the T allele. The PCR condition for eNOS amplification was performed in a total volume of 15 µl reaction mixture containing 2 µl template DNA, 2 µl of each inner primers and 0.5 µl of each outer primers, 5 µl of PCR premix (Taq DNA Polymerase 2 × Master mix RED) and 3 µl of sterile water. The PCR reaction condition for the detection of the variant rs1799983 polymorphism was 95°C for 2 min followed up by 30 cycles, in addition to denaturation at 95°C for 20 s, annealing at 69°C for 20 s, 72°C for 1 min and a final extension at 72°C for 5 min to complete the extension of all PCR fragments. The PCR products were analyzed by electrophoresis on a 2% agarose gel, with DNA marker 100–1500 bp (Cat no: DM001-R500). Figure 1 represents the agarose gel electrophoresis result of 894G > T *eNOS* gene polymorphism.

TGFβ1 C915G (1800471) genotyping

The PCR product sizes for the variant rs1800471 polymorphism were as: 304 bp for two outer primers (control bands), 200 bp for the G allele, and 160 bp for the C allele. The PCR condition for TGFβ1 amplification was performed in a total volume of 15 µl reaction mixture containing 2 µl template DNA, 2 µl of each inner primers and 0.5 µl of each outer primers, 5 µl of PCR premix (Taq DNA Polymerase 2 × Master mix RED) and 3 µl of sterile water. The reaction condition was set as follows: number of cycles 35, initial denaturation (95°C for 2 min), denaturation (95°C for 20 s), annealing temperatures (66°C and 68°C for 1 min), elongation (72°C for 50 s) and final elongation at 72°C for 5 min. The bands of amplified products were identified at three different types of genotypes were attained and observed as bands among which 304 bp and 200 bp for homozygous wild type, 304 bp and 160 bp for homozygous mutant and 304 bp, 200 bp, and 160 bp for heterozygous mutant. The PCR products were analyzed by electrophoresis on a 2% agarose gel with DNA marker 100–1500 bp (Cat no: DM001-R500). Figure 2 shows the gel electrophoretic results for 915G > C TGFβ1 gene polymorphism. The primers used for amplification of eNOS (894 G > T) and TGF-β1 (915 G > C) are listed in Table 1. The table shows the primers designed to target the selected gene polymorphism for the T-Arms PCR.

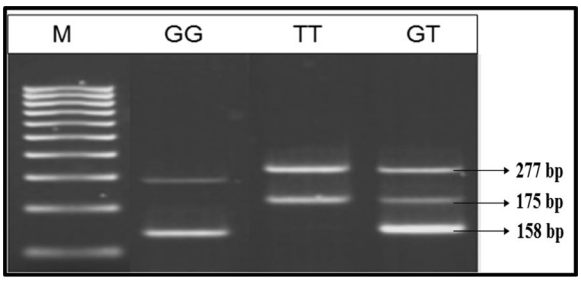

Fig. 1 Gel picture illustrating eNOS G/T polymorphism. Lane 1: DNA ladder (100 bp) Lane 2: GG genotype, Lane 3: TT genotype and Lane 4: GT genotype

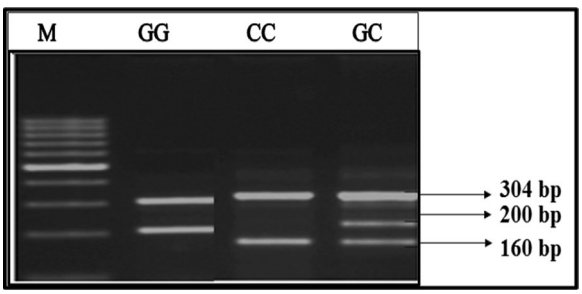

Fig. 2 Gel picture illustrating TGFβ1 G/C polymorphism. Lane 1: DNA ladder (100 bp) Lane 2: GG genotype, Lane 3: CC genotype and Lane 4: GC genotype

Table 1 Designed primer sequences used in tetra arms PCR genotyping

Gene polymorphism	Primer sequence (5′–3′)	Melting temp (°C)	Amplicons length (bp)
eNOS (rs1799983) 894G > T	Forward outer primer		
	GAGGAGGGCATGAGGCTCAGCCCCAGAA	63	
	Reverse outer primer		277
	GGATCAGCACCCCCTTGCAGGCCCTTCT	63	
	Forward inner primer (G allele)		
	CCCCTGCTGCTGCAGGCCCCAGATAAG	65	175
	Reverse inner primer (T allele)		
	CGGGGGGCAGAAGGAAGAGTTCTGGGAGA	65	158
TGFβ1 (rs1800471) 915 C > G	Forward outer primer		
	CCTCCCCACCACACCAGCCCTGTTCG CG	62	
	Reverse outer primer		304
	GTACAGGGCGAGCACGGCCTCGGGCAGC	62	
	Forward inner primer (G allele)		
	GCTGTGGCTACTGGTGCTGACGCCTGGGCG	66	200
	Reverse inner primer (C allele)		
	GCAGGTGGATAGTCCCGCGGCCGCCG	66	160

Validation assay of the genotyping results

The validation of the genotyping results was done by using the DNA sequencing method. Random DNA samples were taken for sequencing and for this the outer primers both forward and reverse primers were taken applicably to amplify the region of each SNP. The reaction used for the amplification of the specified region was executed in a total reaction mixture of 25 µl. To verify the genotyping results random samples were selected for sequencing analysis by the Sanger sequencing method.

Statistical analysis

The statistical analysis was performed by using the SPSS version 21 software for Windows (IBM Analytic, USA). The significance of demographic information among the cases and healthy controls were done by the Chi-square test. The association among the two genetic polymorphisms and their susceptibility to DN was evaluated by the Odds ratio and 95% confidence interval (CI) under four diverse genetic models which involved the co-dominant model, dominant model, recessive model, and the allelic model. A significant difference was assumed if the two-sided p-value ≤ 0.05. Hardy–Weinberg equilibrium (HWE) was assessed by the χ^2 test to determine the distribution of polymorphism among the controls.

Results

Demographic characteristics of the study population

The demographic and biochemical parameters of all the study participants included in the case–control study are presented in Table 2. This table displays the clinical and anthropometric levels of all the three different groups of participants involved in the study. A whole of 420 participants were involved in the study, out of which there were 197 (47%) men and 223 (53%) women with a mean age 55.6 ± 6.36 and the mean BMI was 24.0 ± 3.6. The subjects were divided into three different groups as the T2DM patients with and without DN and control groups each comprising of 140 participants per group. The levels of fasting and postprandial blood glucose, Serum creatinine, LDL, SBP, and DBP were significantly ($p < 0.05$) greater in the T2DM patients with the DN group in comparison to the controls. Whereas eGFR was observed to be reduced in the T2DM with diabetic nephropathy group than the other two groups ($p < 0.05$).

Genotypic frequency distribution between T2DM with and without DN in eNOS and TGF-β1 gene polymorphisms

No substantial deviations from Hardy–Weinberg equilibrium (HWE) ($p > 0.05$) were found in the genotypic distributions among the variants in either of the study groups. The genotype and allelic frequencies of both rs1799983

Table 2 Demographic and clinical characteristics of the study subjects

Characteristics	T2DM patients with DN (*N* = 140)	T2DM patients without DN (*N* = 140)	Control (*N* = 140)	*P* value
Gender (Male:Female)	75:65	62:78	60:80	0.91
Age (years)	56.2 ± 5.7	55.5 ± 6.6	55.1 ± 6.8	0.44
BMI (kg/m^2)	24.0 ± 4.1	24.0 ± 3.4	24.2 ± 3.5	0.56
Diabetes duration (years)				
3–5 years	38	32	–	
5–10 years	34(11.4 ± 4.0)	46 (11 ± 3.8)	–	0.45
> 10 years	68	62	–	
Fasting blood glucose (mg/dL)	156.0 ± 12.7	151.1 ± 4.3	87.9 ± 8.9	< 0.001
Postprandial glucose (mg/dL)	171.3 ± 53.3	164.8 ± 42.7	118.9 ± 12.3	< 0.001
HbA1c (%)	9.0 ± 2.2	8.9 ± 1.9	7.8 ± 2.0	0.68
Protein-to-creatinine ratio (mg/g)	62.8 ± 5.7	54.3 ± 2.5	32.4 ± 1.23	< 0.001
Serum creatinine (mg/dL)	2.80 ± 2.7	0.9 ± 0.24	0.8 ± 0.15	< 0.001
HDL (mg/dl)	64.1 ± 15.9	61.05 ± 18.08	49 ± 15.7	0.08
LDL (mg/dl)	97.2 ± 29.2	95.1 ± 30.3	93.6 ± 31.0	0.96
eGFR (ml/min)	55 ± 13.1	101.1 ± 12.3	130.8 ± 16.0	< 0.001
SBP (mmHg)	145.7 ± 5.5	135.08 ± 7.1	113 ± 7.3	< 0.001
DBP (mmHg)	104.8 ± 4.9	90.3 ± 5.2	75.2 ± 5.4	< 0.001

Data are in mean ± SD

BMI body mass index, *HbA1c* glycosylated haemoglobin, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *eGFR* estimated glomerular filtration rate, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

$p < 0.05$ indicates statistical significance

Table 3 Association of eNOS and TGF-β1 gene polymorphisms in T2DM patients with and without DN

Gene(rsID)	Models	T2DM patients with DN *N* = 140	T2DM patients without DN *N* = 140	Odds ratio (95% CI)	*P* value
	Co dominant model				
	GG	73	80	1.00	–
	GT	24	32	0.69 (0.38–1.26)	0.23
	TT	45	28	1.89 (1.09–3.26)	0.02*
eNOS (rs1799983)	Dominant model-GT + TT vs GG	78	65	1.45 (0.90–2.32)	0.12
	Recessive model-TT vs GG + GT	87	60	2.18 (1.35–3.53)	0.001*
	Allelic model				
	Allele G	177	212	1.00	–
	Allele T	103	68	0.55 (0.39–0.79)	0.001*
	Co dominant model				
	GG	85	82	1.00	–
	GC	29	30	0.95 (0.53–1.70)	0.87
	CC	26	28	0.91 (0.50–1.65)	0.76
TGF-β1 (rs1800471)	Dominant model-GC + CC vs GG	57	62	0.86 (0.53–1.38)	0.54
	Recessive model-CC vs GG + GC	68	73	0.87 (0.54–1.38)	0.56
	Allelic model				
	Allele G	180	176	1.00	–
	Allele C	100	104	1.06 (0.75–1.50)	0.72

eNOS endothelial nitric oxide, *TGF-β1* transforming growth factor

*$p < 0.05$

and rs1800471 polymorphisms were evaluated by using odds ratio and (95%CIs) confidence intervals and the *p*-value as shown in Table 3. The abovementioned table displayed the positive association of rs1799983 polymorphism and a negative association of rs1800471 polymorphism in the comparison between diabetic patients with and without nephropathy.

The analysis of rs1799983 polymorphism demonstrated a positive association of T2DM with and without DN. In the current study, T2DM subjects with DN and TT genotype have a 2.40 increased risk to develop diabetic nephropathy. The TT vs GG + GT genotype had an odds ratio (OR) and a 95% confidence interval of 0.34(0.15–0.74) under a recessive model ($p = 0.007$). The wild type T allele frequency also presented a significant association among T2DM patients with and without DN, with an odds ratio of 1.81 (1.19–2.75), $p = 0.004$. Whereas the analysis of rs1800471 polymorphism showed no significant dissimilarities among the T2DM patients with and without DN in the dominant and recessive models.

Genotypic frequency distribution between T2DM with DN and Controls in *eNOS* and *TGF-β1* gene polymorphisms

The genotypic and allelic frequencies of both rs1799983 and rs1800471 polymorphisms in T2DM patients with DN and controls were performed as shown in Table 4. The abovementioned table displayed the positive association of rs1799983 polymorphism and a negative association of rs1800471 polymorphism in the comparison between diabetic patients with diabetic nephropathy and healthy controls. The frequency distribution in the control group was in accordance with the HWE ($p > 0.05$). Further, the genotypic model of rs1799983 showed OR = 3.31(1.46–4.10) and p-value = 0.004 whereas the allelic model showed OR = 2.01(1.32–3.08) p-value = 0.001. Likewise, the dominant and recessive models indicated OR = 1.45(0.84–2.49) p-value = 0.17 and OR = 0.12(0.04–0.38) p-value = 0.0002, respectively. The genotypic, allelic, and dominant models of rs1799983 polymorphism showed a strong positive association in T2DM patients with DN in comparison with the control however, the dominant model showed a negative association in the diabetic patients with DN as compared with control with $p \geq 0.05$. Also, the analysis of rs1800471 polymorphism showed a negative association in the diabetic patients with DN and control by showing a p-value ≥ 0.05 among the genotypic, allelic, dominant, and recessive genetic models, respectively.

Table 4 Association of eNOS and TGF-β1 gene polymorphisms in T2DM patients with DN and controls

Gene(rsID)	Models	T2DM patients with DN *N* = 140	Controls *N* = 140	Odds ratio (95% CI)	*P* value
	Co dominant model				
	GG	71	82	1.00	–
	GT	36	42	0.80 (0.47–1.36)	0.42
	TT	33	16	2.39 (1.24–4.58)	0.008*
eNOS (rs1799983)	Dominant model- GT + TT vs GG	76	64	1.41 (0.88–2.25)	0.15
	Recessive model-TT vs GG + GT	86	67	1.73 (1.07–2.79)	0.02*
	Allelic model				
	Allele G	177	225	1.00	–
	Allele T	103	55	0.42(0.28–0.61)	0.0001*
	Co dominant model				
	GG	85	79	1.00	–
	GC	29	32	0.88 (0.49–1.55)	0.66
	CC	26	29	0.87 (0.48–1.57)	0.65
TGF-β1 (rs1800471)	Dominant model-GC + CC vs GG	76	83	0.81 (0.50–1.30)	0.39
	Recessive model-CC vs GG + GC	62	74	0.70 (0.44–1.13)	0.15
	Allelic model				
	Allele G	180	171	1.00	–
	Allele C	100	109	1.14 (0.81–1.61)	0.43

eNOS endothelial nitric oxide, *TGF-β1* transforming growth factor

*$p < 0.05$

Genotypic frequency distribution between T2DM patients without DN and controls in *eNOS* and *TGF-β1* gene polymorphisms

The genotypic and allelic frequencies of both rs1799983 and rs1800471 polymorphisms in T2DM patients without DN and controls were performed as shown in Table 5. The abovementioned table displayed the positive association of rs1799983 polymorphism and a negative association of rs1800471 polymorphism in the comparison between diabetic patients without diabetic nephropathy and healthy controls.

The frequency distribution in the control group was in accordance with the HWE ($p > 0.05$). Further, the genotypic model of rs1799983 showed a negative association in the diabetic patients without DN when compared with the control group exhibiting a p-value of ≥ 0.05 in all the models such as allelic, dominant, and recessive. Furthermore, the analysis of rs1800471 polymorphism also showed a negative association in T2DM patients without DN and control by showing a p-value of ≥ 0.05 among all the genetic models. The Table 6 shows a positive association of rs1799983 polymorphism, systolic and diastolic blood pressure and diabetic duration which specified an increased risk of DN in diabetic patients. Whereas, rs1800471 did not show any significant effects. The Table indicates positive association of diabetes duration ($p < 0.01$), systolic blood pressure ($p < 0.03$), diastolic blood pressure ($p < 0.01$), and rs1799983 demonstrated a $p < 0.01$ which showed an increased risk of DN in diabetic patients.

Table 6 Logistic regression analysis for the association among diabetic nephropaathy, genetic polymorphisms and potential risk factors in type 2 diabetes patients

Variables	Odds ratio	95% CI	*P* value
Age	1.03	0.43–1.08	0.94
Diabetes duration (years)	1.32	0.67–2.26	0.01*
BMI	0.76	0.23–0.98	0.57
FBG (mg/dl)	1.26	0.63–2.02	0.35
PPG(mg/dl)	1.22	0.94–3.12	0.06
Smoking	0.97	0.35–1.23	0.21
Systolic blood pressure	1.45	0.87–2.85	0.03*
Diastolic blood pressure	1.20	0.76–1.53	0.01*
Serum creatinine(mg/dl)	0.88	0.58–1.44	0.26
Protein-to-creatinine ratio(mg/g)	1.51	0.61–2.00	0.08
eGFR(ml/min)	1.27	0.82–2.61	0.10
rs1799983 genotype(GT)	1.78	1.18–3.24	0.01*
rs1800471 genotype (GC)	0.40	0.34–1.15	0.12

BMI body mass index, *FBG* fasting blood glucose, *PPG* postprandial blood glucose, *eGFR* estimated glomerular filtration rate, *OR* odds ratio, *CI* confidence interval

Table 5 Association of eNOS and TGF-β1 gene polymorphisms in T2DM patients without DN and Controls

Gene(rsID)	Models	T2DM patients without DN $N = 140$	Controls $N = 140$	Odds ratio (95% CI)	*P* value
	Co dominant model				
	GG	80	82	1.00	–
	GT	38	42	0.86 (0.51–1.46)	0.59
	TT	22	16	1.44 (0.72–2.88)	0.29
eNOS (rs1799983)	Dominant model-GT + TT vs GG	63	59	1.12 (0.70–1.80)	0.62
	Allelic model				
	Allele G	212	225	1.00	–
	Allele T	68	55	0.76 (0.50–1.13)	0.18
	Co dominant model				
	GG	82	79	1.00	–
	GC	30	32	0.92 (0.52–1.61)	0.77
	CC	28	29	0.95 (0.53–1.71)	0.88
TGF-β1 (rs1800471)	Dominant model- GC + CC vs GG	62	67	0.86 (0.54–1.38)	0.54
	Recessive model-CC vs GG + GC	45	57	0.68 (0.42–1.12)	0.13
	Allelic model				
	Allele G	176	171	1.00	–
	Allele C	104	109	1.07 (0.76–1.51)	0.66

eNOS endothelial nitric oxide, *TGF-β1* transforming growth factor

*$p < 0.05$

Table 7 Characteristics and clinical parameters of different eNOS genotypes

Clinical characteristics	eNOS GG	eNOS TT	*P* value
Age (years)	55.5 ± 0.61	55.1 ± 0.35	NS
Gender (male/female)	43/64	52/89	NS
BMI (kg/m^2)	25.1 ± 1.11	24.9 ± 1.09	NS
Diabetes duration (years)	15.3 ± 0.4	12 ± 0.1	< 0.001
Fasting blood glucose (mg/dL)	135.9 ± 0.66	141.8 ± 2.60	0.0007*
Post prandial glucose (mg/dL)	123.4 ± 1.48	125.8 ± 2.16	0.03*
HbA1c (%)	7.6 ± 2.2	8.6 ± 1.2	< 0.05*
eGFR (ml/min)	73.5 ± 1.38	66.1 ± 2.1	0.03*
Protein-to-creatinine ratio (mg/g)	49.3 ± 2.4	62.3 ± 3.2	< 0.001*
Systolic blood pressure (mmHg)	115 ± 0.80	125.8 ± 0.8	< 0.001*
Diastolic blood pressure (mmHg)	78.7 ± 1.43	83.0 ± 4.12	0.01*

Data are *n*, means ± SEM

BMI body mass index, *HbA1c* glycosylated haemoglobin, *eNOS* endothelial nitric oxide, *eGFR* estimated glomerular filtration rate, *NS* non-significant

*$p < 0.05$

Clinical characteristics of *eNOS* and *TGF-β1* genotypes

Table 7 illustrates the clinical as well as biochemical features of T2DM patients with and without DN in different genotypes of *eNOS* gene polymorphism. As table indicates a positive association with $p < 0.01$ was shown by several parameters like diabetes duration, FBS, PPG, HbA1c, eGFR, PCR, systolic and diastolic blood pressure in association with increased risk of DN in diabetic patients with the presence of TT genotype of rs1799983 polymorphism. A substantial difference was found among the diabetic patients with and without DN which was confirmed by the frequencies of the wildtype GG and heterozygous frequencies. It was observed that despite the factors such as less duration of diabetes, levels of glucose, HbA1c, eGFR, protein creatinine ratio, systolic blood pressure, diastolic blood pressure, the renal function declined more rapidly in the diabetic patients with diabetic nephropathy and the TT heterozygous genotype when compared with the wildtype GG genotype. The frequency of TT genotype was considerably higher in the diabetic patients with DN in comparison to those patients without DN. However, no significance was found among the other factors such as age, gender, and BMI. Whereas the T2DM patients with DN and *TGFβ1* (G/C) genotypes, did not show any significance in the clinical as well as the biochemical characteristics (data not shown).

Discussion

The present study has examined the role of *eNOS* gene variants and *TGFβ1* variants with the risk of progression of nephropathy in DN patients with type 2 diabetes. The results have shown a strong association between diabetic patients with DN (p value < 0.05) signifying that eNOS variants can be a possible risk factor in the development of diabetic nephropathy in type 2 diabetic patients. Further, no significant results were obtained amongst the *TGFβ1* variants and diabetic nephropathy in the study population. Existing studies have shown that an abnormal *eNOS* activity produced because of the mutation could be associated with various pathological conditions like hypertension, and atherosclerosis, which may further deteriorate the endovascular injury in diabetic patients. *eNOS* is likely to enhance the sensitivity of glomerular disease in the metabolic environment of diabetes and propose that this pathway can be convoluted in the renal complications of type 1 as well as type 2 diabetic patients. Moreover, polymorphisms in the *eNOS* gene expression are proved to be linked with diabetic nephropathy among diabetes patients. The mechanism which is responsible for this probable association is still unknown. Nevertheless, the genetic variants of *eNOS* are known to be the basis of a defective synthesis of nitric oxide at a low level thereby intensifying the predisposition to glomerular disease and therefore decline the renal function [17]. Hence there is a possibility that the metabolic pathway of diabetes can intricate in the renal complications as well. Our results were supported by the outcomes of a meta-analysis by Varghese et al., as their findings suggested a positive association of rs1799983 polymorphism with a higher risk of diabetic nephropathy [18]. These results are also in agreement with the conclusions of Sahar et al.,Shin Shin et al., and Neugebauer et al., who proved the association of *eNOS* polymorphism in the advancement of diabetic nephropathy in Korean and Japanese T2DM patients, respectively [19–22]. The results with reference to rs1799983 have shown a strong association of the TT genotype and T allele between DN patients and T2DM with an odds ratio of 1.89 (1.09–3.26) and a p value = 0.02 signifying that mutant T allele can be a possible risk factor in the advancement of diabetic nephropathy in type 2 diabetic patients. The study by Mohd et al., 2019 stated a highest odds ratio 2.39 (1.17–4.88) among the other studies, and thereby showed a wide-range of confidence interval which suggested small sample size or other factors leading to probable bias. In contrast with the aforementioned study, even though the present study resulted in a lesser odds ratio and low range of confidence interval but exhibited a significant difference along with a higher heterogeneity towards increased risk.

Mohd et al., have reported the case–control association study, in order to assess the influence of four different oxidative stress-related gene polymorphisms including *NOS3* rs1799983 polymorphism as risk factors in the progression of diabetic nephropathy in Malaysian

diabetic patients [23]. Their findings demonstrated that the T allele of *NOS3* gene polymorphism is significantly linked with the development of DN specifically amongst the Chinese and Indian populations included in the study. A recent study by Shoily et al., 2021 have proved the association of rs1799983 polymorphism of NOS3 to have positive link specifically in the populations such as European and Americans. They have demonstrated the role of *NOS3* genetic polymorphism in the progression of diabetes and diabetic complications such as diabetic nephropathy, retinopathy etc. [24].

With respect to the *TGFβ1* (−915G>C) polymorphism, the present study did not find any significant association in the advancement of diabetic nephropathy. The results with reference to rs1800471, the CC genotype have shown an OR and 95% CI of 0.91 (0.50–1.65) non-significant risk displaying a $p=0.76$ in the T2DM patients with and without DN. These results are in agreement with the findings of El-Sherbini et al., where they found insignificant dissimilarities in the alleles and genotypic frequencies of *TGFβ1* (G915C) polymorphism in Egyptian type 2 diabetic patients [25]. McKnight et al., and Zhang et al., have also indicated the insignificant results of *TGFβ1* variants with the risk of DN among the Asian and Caucasian population respectively [26, 27]. Nevertheless, opposing to these results Valladares et al. (2010) have reported a positive association of T869C and G915C polymorphisms of the TGFβ1 gene with DN. They also stated that lower levels of triglycerides, as well as cholesterol, were noticed in subjects with TT homozygotes for the T869C polymorphism. They reported a highest OR of 4.16 (2.92–5.94) among the other studies, which was also comparatively very high than our study [28]. Another recent meta-analysis study by Mai et al., 2020 has also proved the association of *TGFβ1* polymorphism (rs18000471) in the development of chronic kidney diseases [29].

Such difference between the results is based on the ethnicity of different study populations. These genetic variances will be due to different lifestyles as well as diverse linkage maps. The ethnicity-specific association studies are required to elucidate the population stratification of diabetic nephropathy and related risk factors.

Conclusion

In conclusion, this case–control study exhibits a significant association of *eNOS* gene variant (rs1799983) in the development of DN in type 2 diabetic patients. This investigation further reported a non-significant association of the *TGFβ1* gene variant (rs1800471) in the progression of diabetic nephropathy in the studied population. This association study can probably assist in identifying the genotypes of the indicated genes in patients with renal disorders who are at high risk of developing diabetic nephropathy. Though these findings alone will not be sufficiently adequate to get a piece of conclusive evidence primarily because of the small sample size of the studied population. Still, it could be considered as strong evidence with significant information on the influence of the investigated genetic variants on the susceptibility to diabetic nephropathy. Further, it is essential to enlarge the sample size and combine it with other risk factors such as lifestyle changes in order to increase the knowledge on more issues influencing the advancement of diabetic nephropathy.

Abbreviations

DN: Diabetic nephropathy; T2DM: Type 2 diabetes mellitus; ESRD: End-stage renal disease; eNOS: Endothelial nitric oxide synthase; TGF-β1: Transforming growth factor-beta 1; Tetra-ARMS-PCR: Tetra-primer amplification refractory mutation system PCR; KDOQI: Kidney Disease Outcomes Quality Initiative; HWE: Hardy–Weinberg equilibrium.

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Authors' contributions

The first author (S.V) carried out the genotyping studies, interpretation of the data and performed statistical analysis and drafted the manuscript. G.K: revised the final draft of the manuscript and approved for further procedures. Both authors read and approved the final manuscript.

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Availability of data and materials

The datasets analysed during the current study are available with the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The current study was approved by the Institution's Ethical Committee of Chettinad Academy of Research and Education on 19 November 2017 with an approval number (IHEC No 366/IHEC/10-17). A written informed consent form was attained from all participants involved in the study.

Consent for publication

The consent for publication were obtained from all the participants enrolled in the study.

Competing interests

All the authors state that there is no conflict of interest.

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