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COMPREHENSIVE ANALYSIS OF VARIOUS GENES IMPLICATED IN REGULATION OF DIABETIC COMPLICATIONS: A CROSS-SECTIONAL STUDY

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Abstract

Objective: Diabetic nephropathy (DN) and diabetic retinopathy (DR) are two of the most devastating microvascular complications caused by hyperglycaemia. The current study aims to investigate the co-existence of DN and DR in the polymorphism as well mRNA expression for the following genes: angiotensin converting enzyme (ACE), angiotensinogen (AGT), receptor for advanced glycation end products (RAGE), aldose reductase (ALR2), and vascular endothelial growth factor (VEGF).

Material and Method – This current study included 60 DN patients. All participants were divided into two groups: Group 1 (DN+DR) and Group 2 (DN-DR). Polymorphism in all genes was identified by polymerase chain reaction (PCR), followed by restriction enzyme. The SPSS version 26.0 software was used to analyse biochemical parameters and the relationship of gene polymorphisms. The Quantitative Reverse Transcriptase PCR (qRT-PCR) amplification method was employed to assess mRNA expression in all genes. The 2DDCt approach was utilized to evaluate the relative expression levels of the ACE, AGT, RAGE, ALR2, and VEGF genes.

Results- The relative mRNA expression of the VEGF gene was found to be significantly higher in the DN+DR group than in the DN-DR group. The association between VEGF gene polymorphism and its relative mRNA expression in the DN+DR group was substantially greater in the ID (p=0.03) and DD (p=0.01) genotypes than in the DN-DR group.

Conclusion- The association of VEGF gene polymorphism and its relative mRNA expression levels with the prevalence of diabetic retinopathy was significant. It suggests that the VEGF gene has a unique function in DR patients with DN.

Keywords: Diabetes, Diabetic nephropathy, Gene expression, PCR

INTRODUCTION

Diabetes mellitus is the most common endocrine system illness. Diabetes has been on the rise in recent years, and it has emerged as one of the key contributors to a variety of health conditions that have a significant influence on the global economy. According to the International Diabetes Federation's (10th edition), there are presently 537 million individuals worldwide who suffer with diabetes. The IDF committee also predicted that the number of diabetic patients will increase to 643 million by 2030 and 783 by 2045 (1). The number of diabetic patients in India is expected to reach 69.9 million by 2025 (2).

A large number of individuals are still erroneously which is mostly due to a lack of physical exercise, an unbalanced diet, which leads to obesity, and an irregularity in insulin secretion. According to many studies, one of the primary causes of DM-induced organ dysfunction is the advancement of comorbidities such as nephropathy or diabetes kidney disease (DKD), retinopathy, and neuropathy etc. (3). Diabetic nephropathy (DN) and retinopathy (DR) are maybe the two most dreaded. Both disorders cause significant morbidity and mortality, as well as end-stage renal disease (ESRD) and blindness. DN can be recognized by chronic microalbuminuria (30-299 mg/g creatinine), which develops to macroalbuminuria (300 mg/g creatinine) and a decrease in glomerular filtration rate (GFR) (4). Aneurysms, neovascularization with poorly formed weak arteries, vascular rupture, and retinal hemorrhage are all symptoms of diabetic retinopathy (DR). Although other variables such as a family history of diabetes, hypertension, and diabetes duration may lead to the development of these problems. However, retinopathy does not affect all DN patients. Diabetes retinopathy is currently known to affect just one-third of diabetic nephropathy patients (4, 5). Common underlying genetic variables may play a crucial role in defining the relationship of DR and DN in the DN patients.

Several studies have recently revealed a link between hyperglycemia and the mRNA expression of renin-angiotensin system (RAS), receptor for advanced glycation end products (RAGE), aldose reductase (ALR2), and vascular endothelial growth factor (VEGF) genes (6, 7). Angiotensin converting enzyme (ACE) is a critical enzyme in the RAS system because it initiates the synthesis of angiotensin II, which regulates blood pressure and water salt balance. Previous research has suggested that the ACE gene and ACE inhibitor is linked to DN (8, 9). In a few studies, another RAS group gene i.e., angiotensinogen (AGT), had comparable results. Advance glycation end products (AGEs) are formed by the nonenzymatic glycation of protein followed by oxidation processes (10). The buildup of AGEs in DM patients' kidneys and other organs has been linked to the development of DN and vasculopathy (11). Another study found that stimulating aldose reductase (ALR2) in the polyol pathway causes glucose to be converted to sorbitol. In diabetes, sorbitol builds up in the cell, producing osmotic stress. As a result, the polyol pathway has been associated to diabetic microvascular sequelae such as nephropathy and retinopathy, in addition to ALR anthoserosis (11, 12, 13). VEGF is a potent vasoactive cytokine that is overexpressed in diabetes and increases vascular permeability in the retina as well as kidneys (6, 14).

We studied common underlying genetic characteristics that may play an essential role in defining the connection between DR and DN in diabetic nephropathy patients by assessing the expression of five genes, ACE, AGT, RAGE, ALR2, and VEGF. Until now, relatively few comparative studies on the co-existence of DN and DR in diabetic patients have been reported.

MATERIALS AND METHODS

Study design and subjects

The purpose of this our cross-sectional study was to see if similar underlying genetic characteristics contribute to the relationship of retinopathy and nephropathy in type 2 diabetic nephropathy patients. A total of 60 individuals with diabetic nephropathy were included (from October 2019 to August 2022) in the study and divided into two groups: Group 1 patients (n=30) had diabetic nephropathy with retinopathy (DN+DR), whereas Group 2 patients (n=30) had diabetic nephropathy without retinopathy (DN-DR). The research was carried out at the outpatient clinics of the University College

of Medical Sciences' (UCMS) Centre for Diabetes, Endocrinology, and Metabolism (DEM) and GTB Hospital in Delhi, India. The current study has been approved by the UCMS Institutional Ethics Committee - Human Research (IEC-HR) (Ref.No., IEC-HR/2019/38/4R). T2DM patients aged 20-70 years with chronic microalbuminuria (30-300 mg/g creatinine) or macroalbuminuria (>300 mg/g creatinine), with or without diabetic retinopathy were included. Subjects who were using nonsteroidal anti-inflammatory medicines (NSAIDs), nephrotoxic drugs, or experienced a urinary or systemic infection were excluded from the research. An ophthalmologist examined all recruited participants for diabetic retinopathy using a direct fundoscopic examination. Diabetes Mellitus was diagnosed in the study's patients using the ADA 2017 (15).

Sample collection and genomic DNA extraction

Patients peripheral venous blood (5ml) was collected in ethylene diamine tetra acetic acid (EDTA)-coated tubes for genomic DNA extraction. The DNA was obtained from the patient's blood using the QIAamp DNA mini kit (Qiagen) and kept at -80 °C until further molecular investigation. The isolated DNA had an optical density close to 1.8 and was considered pure DNA.

Gene polymorphism analysis:

Primer designing

All selected genes full domain sequences were acquired from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed). Primer 3 software (http://primer3.ut.ee/) was used to build primers for genotyping analysis. The generated sequences were then examined for the possibility of hairpin formation and self-complementarity (http://www.basic.northwestern.edu /biotools/ oligocalc.html). Table 1 display the primers for polymorphism and expression used in this study. With the exception of the ACE gene, the same primers were used for both polymorphism and expression.

Determination of genotypes

Polymorphisms in the genes ACE (I/D), AGT (M235T), RAGE (gly82ser), ALR2 (C-108T), and VEGF (I/D) were investigated using polymerase chain reaction (PCR) amplification of extracted DNA and restriction endonuclease digestion. After initial denaturation for 5 min seconds at 95°C, 35 amplification cycles were performed with a PCR (Bio-Rad T100TM Thermal Cycler) using the pre-set programme [initial denaturation for 30 sec at 95°C followed by annealing for 60 sec at 62.3ºC for ACE (I/D), 65.7 ºC for AGT(M235T), 56ºC for RAGE (gly82ser), 66 ºC for ALR2(C-106T), 59ºC for VEGF (I/D) and extension for 1 min at 72°C with final extension at 72ºC for 10 min]. 100 ng of genomic DNA and 10 pmol primer were added in a 25 µL PCR reaction mixture. The PCR master mix included 0.2 µL of 5U Taq DNA polymerase, 0.5 µL of each dNTP, 1X PCR buffer, and 3 mM MgCl2. By adding nuclease free water, the amount of the master mix up was increased to 22µL. Each reaction comprised 22 µL of master mix and 3µL of DNA solution (50-150ng/L). The ACE gene was amplified in two steps using a set of flanking primers that differentiate between insertion-specific sequences. The PCR product was subsequently digested with restriction enzymes for AGT (Tth111I) (NEB), ALR2 (BfaI) (NEB), and RAGE (AluI) (NEB) at 37°C for 16 hours to detect the M/T, C/T, and G/S polymorphisms, respectively. The digested DNA fragments were electrophoresed on a agarose gel (2%) and visually analysed using a UV transilluminator.

Gene Expression analysis:

mRNA extraction and cDNA formation

To isolate RNA from whole blood, 750 L of Trizol® LS reagent (Invitrogen, life technologies, USA) was added to the 250 L of whole blood in the microcentrifuge tube. For adequate cell lysis, the solution was well mixed using a pipette and kept at -80 °C for the purpose of molecular studies. A modified Chomcznski and Sacchi technique was used to isolate total RNA. The total RNA yield was of high quality (A 280/260 was 1.90-1.95). For reverse transcription of total RNA, a cDNA synthesis kit (iScriptTM cDNA synthesis) was used. This kit delivers a sensitive and simple two-step reverse transcription quantitative PCR solution. Only 0.1g to 0.5g of total RNA was utilized per sample for

cDNA synthesis. We employed a Thermal Cycler (Bio-Rad T100TM) for cDNA amplification, and the reaction was run under the following conditions: Priming at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, and reverse transcription inactivation (RT) at 95°C for 1 minute. This whole solution was then either utilized as a template sample for qRT-PCR or kept at -80°C for long-term storage.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

SSO FastTM EvaGreen Supoermix (1X) reagent kit and qRT-PCR (Biorad) equipment was used for qRT-PCR amplification. The qRT-PCR assays were carried out in triplicate. The basic thermocycler programming involves an initial denaturation at 95℃ for 3 minutes, followed by 35 cycles of denaturation at 95 ℃ for 1 minute (the specific annealing temperature is provided in Table-1). Endogenous controls were GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and B2M (2-microglobulin); the relative expression level of 5 genes was calculated using the 2DDCt method.

Statistical analysis

The Department of Biostatistics, UCMS, and GTB Hospital performed the statistical analysis for the current study. SPSS version 26.0 was used for statistical analysis. The differences between the two groups were examined using an independent Student's t-test. The Hardy-Weinberg equation and the Chi-square test were used to compare the polymorphism distributions between the two groups. The Chi-square test was performed to assess the significance of the demographic, biochemical, and relative mRNA expression data between the two groups. Less than 0.05 p-values were considered statistically significant.

RESULTS

Demographic and biochemical characteristics of the study population

The demographic and biochemical parameters of the study subjects included in the both groups are shown in table 2 and 3. In all, 60 people participated in the study. The mean waist circumference of study participants was significantly higher in the DN-DR group as compared to the DN+DR group. Among biochemical measures: the DN-DR group had considerably higher mean eGFR (MDRD), mean eGFR (EPI), and mean haemoglobin and significantly lower mean serum potassium levels and mean HDLc than the DN+DR group.

Genotypic frequency distribution of gene polymorphisms:

Table 4 shows the genotype distribution (n=30) and frequencies of both group for the following genes: ACE (I/D), AGT (M235T), RAGE (gly82ser), ALR2 (C-106T), and VEGF (I/D). Significant differences were observed in the distribution of ID ($p<0.03$) and DD ($p<0.01$) genotypes of the VEGF(I/D) gene polymorphism between two the groups. There were not any significant differences in the genotypic frequency of ACE (I/D), AGT (M235T) and RAGE (gly82ser) gene polymorphisms between the two groups.

Relative mRNA expression of ACE, AGT, RAGE, ALR2 and VEGF gene

The DN+DR group had 5.3-fold greater relative mRNA expression of the VEGF gene than the DN-DR group, which was statistically significant ($p \leq 0.001$). Both groups had similar levels of ACE, AGT, RAGE, and ALR mRNA expression (Table 5).

Association between polymorphisms and mRNA expression of five genes in two groups.

We additionally investigated the relationship between five gene polymorphisms and their relative mRNA expression. We observed that the ID and DD genotypes of the VEGF gene, as well as their relative mRNA expression levels, were strongly linked with the risk of diabetic retinopathy in individuals with nephropathy (Table 6).

DISCUSSION

It is estimated that retinopathy occurs in 80%-90% of diabetic nephropathy patients (16). Diabetic nephropathy (40%) and diabetic retinopathy (27%) affect the majority of chronic diabetes patients around the world (17, 18). Among the well-known pathophysiology aspects of DN and DR are inflammatory processes, oxidative stress, an activated renin-angiotensin-aldosterone system (RAAS), neovascularization, and retinal edema etc. (19). Recently discovered different aspects of DN and DR pathogenesis are podocyte autophagy, mitochondria dysfunction, and certain genetic and epigenetic alterations (20, 21). Despite these results, current understanding regarding the etiology of DN and DR is insufficient, and therapy of this mysterious illness is mostly centered on managing blood pressure, reducing blood glucose, inhibiting the renin-angiotensin system, and so on (22). Consequently, perspectives into the toxicity and genetic etiology of DN and DR may lead to novel therapeutic approaches. Exploring biological variability at the genome level might be beneficial in this context. Numerous genes may undergo synchronized expression changes throughout the development of DN and DR. Until currently, most studies have concentrated on the differential expression of genes linked to DN or DR, but have disregarded their great degree of interconnectedness. By evaluating the polymorphisms and expression levels of five genes (ACE, AGT, RAGE, ALR2, and VEGF) in diabetic nephropathy patients, we investigated common underlying genetic traits that may play an important role in establishing the link between DR and DN. The purpose of this study was to establish the role of five key genes in the etiology of DR in type 2 DN patients.

Among demographic characteristics, DN-DR group had considerably greater waist circumferences (WC) than the DN+DR group. Earlier research suggested that obesity increased the incidence of DR (23), whereas others found no connection (24).

Among various biochemical parameters, we observed a substantial and positive association between the DN+DR group and lower levels of eGFR the strong association of baseline renal profile (serum creatinine, eGFR and albuminuria) with the development of new- onset DR suggests that DN can predict the development of DR. Our study has shown that both eGFR (MDRD and EPI) were significantly lower in DN+DR group as compared to another group. Several cross-sectional studies in India have already studied the relationship between DN and DR. According to Rajalakshmi et al, (2020) (25), there is an inverse connection between eGFR levels and DR at baseline. In accordance with previous finding (26), our DN patients with DR exhibited substantially lower eGFR levels at baseline than those without DR. The DN+DR group also had significantly lower mean hemoglobin levels than the DN-DR group, indicating that diabetic nephropathy patients with retinopathy were more likely to develop anemia. DN + DR group exhibited considerably higher mean HDLc than the DN-DR group. The most intriguing finding in our study was that high HDLc was related with DR. This conclusion contradicts prior research, which found substantial evidence for an independent link of low HDL cholesterol levels with both kidney disease and macroangiopathic consequences of type 2 diabetes (27). Our findings were consistent with those of Sasso et al., 2019 (28), who discovered a link between high HDL cholesterol and DR.

Our study demonstrated a significant association between ID ($p<0.01$) and DD ($p<0.03$) genotypic frequencies of the VEGF (I/D) gene polymorphism and DR risk in patients with diabetic nephropathy. The findings suggested that VEGF (I/D) gene polymorphisms might contribute to the development of retinopathy in DN patients. These results are consistent with those published by Khan et al. (2020) in the Pakistani population (29). They observed that the DD genotype of the VEGF gene polymorphism may be associated to T2DM complications like retinopathy and nephropathy. Other studies additionally found links between the VEGF gene polymorphism and retinopathy in various other populations (30, 31). Our data also demonstrated no associations between ACE, AGT, RAGE and ALR2 gene variants and retinopathy risk in DN patients. The findings are similar with previous observations made by Miura et al. (1999) (32), suggesting that genetic variations in these polymorphisms are unlikely to play a major role in the risk of DR in DN patients.

The relative mRNA expression of the VEGF gene in the DN+DR group was 5.3-fold greater than in the DN-DR group, which was statistically significant. This shows that the VEGF gene has a special function that promotes DR risk in patients with DN. While a few earlier studies (33) found a significant relationship between VEGF gene expression and DN or DR, only one study by Pe'er et al., 1996 (34) represent a clear association between VEGF gene expression and PDR- related neovascularisation. The fact that VEGF gene expression in PDR increases in response to retinal hypoxia suggests that VEGF may be one of the processes connecting ocular ischaemia and angiogenesis in PDR.

A possible reason for the lack of relationship between polymorphism and their similar expression of the ACE, AGT, ALR, and RAGE genes is that polymorphism increases expression in both microvascular complications of DN and DR in similar manners. It might be associated with ethnicity and environmental factors that affect polymorphism and gene expression.
We have also assessed the association between ACE, AGT, RAGE, ALR2, and VEGF gene polymorphisms and their relative mRNA expression, and we found that the ID (p=0.03) and DD (p=0.01) genotypes of the VEGF gene were more expressed in the DN+DR group than in DN-DR group. This suggests that higher expression of the D allele in DN patients is related with a higher risk of DR. However, there was no substantial correlation between ACE, AGT, RAGE, and ALR2 gene polymorphisms and their expression. Buraczynska et al., 2007 (30), discovered that diabetic retinopathy patients with the DD genotype of VEGF gene had higher serum VEGF levels than those with the II genotype, however they have not studied the association of VEGF I/D polymorphism with VEGF mRNA expression. This suggests that VEGF gene overexpression may result in abnormal VEGF mRNA expression, exacerbating the retinopathy in DN patients.
Finally, our data suggest that ID and DD genotypes of the VEGF gene polymorphism, as well as their expression in the promoter region, are associated to the incidence of diabetic retinopathy in type 2 DN patients compared to those without DR. The VEGF genotype (ID and DD) was found to be an independent risk factor for retinopathy in diabetic patients. The presence of the deletion (D) allele is most likely associated with higher transcriptional activity for retinopathy risk. One possible explanation is that persons who have both DR and DN have greater impairments, such as high blood pressure, blurred vision, neovascularization, blindness, hyperkalemia, cardiovascular diseases and end-stage renal illness. Prospective investigations in larger diabetic patient groups, as well as functional research, will be required to determine the relevance of the VEGF I/D polymorphism in diabetes microvascular complications.

LIMITATIONS

We didn't include non-diabetic control or diabetic control without nephropathy in this study due to limited funds. The limited sample size in the study groups was one of the study's limitations. The mRNA expression analysis was performed on patient whole blood rather than kidney and retinal tissue samples.

CONCLUSION –

A higher relative mRNA expression of VEGF gene was associated with the development of retinopathy in diabetic nephropathy population. A higher relative mRNA expression of ID and DD genotype or D allele of the VEGF gene is associated with a higher risk of retinopathy in patients with DN. Future research with a larger sample size is critically required to properly identify and characterize the importance of common genes linked to both serious diabetes consequences and their corresponding disease variations.

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Conflict of Interest- The authors have no conflict of interest.

REFERENCES

1. Sun H, Saeedi P, Karuranga S, Pinkepank M, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes research and clinical practice. 2022; 183:109119.
2. Misra A, Gopalan H, Jayawardena R, Hills AP, et al. Diabetes in developing countries. Journal of diabetes. 2019;11(7):522-39.
3. Ojo O. An overview of diabetes and its complications. Diabetes Research Open Journal. 2016;2(2): e4-6.
4. Kare PK, Aggrawal N, Varshney P, Ghosh R, et al. Screening of Type 2 Diabetes Mellitus Patients for Micro-albuminuria and Its Relationship with Diabetic Retinopathy. International Journal of Biochemistry Research & Review. 2016;14(4):1-9.
5. Rani PK, Raman R, Gupta A, Pal SS, et al. Albuminuria and diabetic retinopathy in type 2 diabetes mellitus sankara nethralaya diabetic retinopathy epidemiology and molecular genetic study (SN-DREAMS, report 12). Diabetology & metabolic syndrome. 2011;3(1):1-8.
6. Konoshita T, Wakahara S, Mizuno S, Motomura M, et al. Tissue gene expression of renin-angiotensin system in human type 2 diabetic nephropathy. Diabetes Care. 2006;29(4):848-52.
7. Bortoloso E, Del Prete D, Dalla Vestra M, Gambaro G, et al. Quantitave and qualitative changes in vascular endothelial growth factor gene expression in glomeruli of patients with type 2 diabetes. European Journal of Endocrinology. 2004;150(6):799-807.
8. Paul M, Wagner J, Dzau VJ. Gene expression of the renin-angiotensin system in human tissues. Quantitative analysis by the polymerase chain reaction. The Journal of clinical investigation. 1993;91(5):2058-64.
9. Aggarwal N, Kare PK, Varshney P, Kalra OP, et al. Role of angiotensin converting enzyme and angiotensinogen gene polymorphisms in angiotensin converting enzyme inhibitor-mediated antiproteinuric action in type 2 diabetic nephropathy patients. World Journal of Diabetes. 2017 ;8(3):112.
10. Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. New England Journal of Medicine. 1988;318(20):1315-21.
11. Ruderman NB, Williamson JR, Brownlee M. Glucose and diabetic vascular disease 1. The FASEB journal. 1992;6(11):2905-14.
12. Kyselova Z, Stefek M, Bauer V. Pharmacological prevention of diabetic cataract. Journal of Diabetes and its Complications. 2004;18(2):129-40.
13. Larkins RG, Dunlop ME. The link between hyperglycaemia and diabetic nephropathy. Diabetologia. 1992;35:499-504.
14. Caldwell RB, Bartoli M, Behzadian MA, El-Remessy AE, et al. Vascular endothelial growth factor and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives. Diabetes/metabolism research and reviews. 2003;19(6):442-55.
15. American Diabetes Association. 1. Promoting health and reducing disparities in populations. Diabetes Care. 2017;40(Supplement_1):S6-10.
16. Rossing K, Christensen PK, Hovind P, Tarnow L, et al. Progression of nephropathy in type 2 diabetic patients. Kidney international. 2004 ;66(4):1596-605.
17. Gheith O, Farouk N, Nampoory N, Halim MA, et al. Diabetic kidney disease: worldwide difference of prevalence and risk factors. Journal of nephropharmacology. 2016;5(1):49.

18. Alemu Mersha G, Alimaw YA, Woredekal AT. Prevalence of diabetic retinopathy among diabetic patients in Northwest Ethiopia—A cross sectional hospital-based study. Plos one. 2022 ;17(1): e0262664.
19. Cahn A, Cernea S, Raz I. The SONAR study—is there a future for endothelin receptor antagonists in diabetic kidney disease? Annals of Translational Medicine. 2019;7(Suppl 8).
20. Zhang Y, Li W, Zhou Y. Identification of hub genes in diabetic kidney disease via multiple-microarray analysis. Annals of Translational Medicine. 2020 ;8(16).
21. Aggarwal N, Kumar Kare P. Diabetes Microvascular Complications: An Overview of Epigenetic Modifications. Type 2 Diabetes - From Pathophysiology to Cyber Systems. United Kingdom: IntechOpen; 2021:171-196.
22. Brenner BM, Cooper ME, De Zeeuw D, Keane WF, et al. Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. New England journal of medicine. 2001 ;345(12):861-9.
23. Van Leiden HA, Dekker JM, Moll AC, Nijpels G, et al. Blood pressure, lipids, and obesity are associated with retinopathy: the hoorn study. Diabetes care. 2002;25(8):1320-5.
24. Wan H, Wang Y, Xiang Q, Fang S, et al. Associations between abdominal obesity indices and diabetic complications: Chinese visceral adiposity index and neck circumference. Cardiovascular Diabetology. 2020; 19:1-2.
25. Rajalakshmi R, Rani CS, Venkatesan U, Unnikrishnan R, et al. Correlation between markers of renal function and sight-threatening diabetic retinopathy in type 2 diabetes: a longitudinal study in an Indian clinic population. BMJ open diabetes research & care. 2020;8(1).
26. Kaewput W, Thongprayoon C, Rangsin R, Ruangkanchanasetr P, et al. Associations of renal function with diabetic retinopathy and visual impairment in type 2 diabetes: A multicenter nationwide cross-sectional study. World journal of nephrology. 2019 ;8(2):33.
27. Sacks FM, Hermans MP, Fioretto P, Valensi P, et al. Association between plasma triglycerides and high-density lipoprotein cholesterol and microvascular kidney disease and retinopathy in type 2 diabetes mellitus: a global case–control study in 13 countries. Circulation. 2014;129(9):999-1008.
28. Sasso FC, Pafundi PC, Gelso A, Bono V, et al. High HDL cholesterol: A risk factor for diabetic retinopathy? Findings from NO BLIND study. Diabetes research and clinical practice. 2019; 150:236-44.
29. Khan SZ, Ajmal N, Shaikh R. Diabetic retinopathy and vascular endothelial growth factor gene insertion/deletion polymorphism. Canadian Journal of Diabetes. 2020;44(3):287-91.
30. Buraczynska M, Ksiazek P, Baranowicz-Gaszczyk I, Jozwiak L. Association of the VEGF gene polymorphism with diabetic retinopathy in type 2 diabetes patients. Nephrology dialysis transplantation. 2007;22(3):827-32.
31. Gupta P, Madhu SV, Bubber P. Genetic polymorphism in patients with diabetic nephropathy and retinopathy: A Cross-sectional Study. Journal of clinical and diagnostic research.2013;17(8):1-7.
32. Miura J, Uchigata Y, Yokoyama H, Omori Y, et al. Genetic polymorphism of renin-angiotensin system is not associated with diabetic vascular complications in Japanese subjects with long-term insulin dependent diabetes mellitus. Diabetes research and clinical practice. 1999;45(1):41-9.
33. Cha DR, Kang YS, Han SY, Jee YH, et al. Vascular endothelial growth factor is increased during early stage of diabetic nephropathy in type II diabetic rats. Journal of Endocrinology. 2004;183(1):183-94.
34. Pe'er J, Folberg R, Itin A, Gnessin H, et al. Vascular endothelial growth factor upregulation in human central retinal vein occlusion. Ophthalmology. 1998 ;105(3):412-6.

Tables:

Table 1: Primers for ACE, AGT, RAGE, ALR2 and VEGF gene for genotyping and quantitative reverse transcriptase PCR

Gene	Primer sequence	Annealing temperature(˚C)
ACE gene (I/D) (Flanking pair)	F:5'CTGGAGACCACTCCCATCCTTTCT 3' R:5'GATGTGGCCATCACATTCGTCAGAT 3'	**62.3**
ACE (I/D) (Insertion-specific pair)	F:5'TGGGACCACAGCGCCCGCCACTAC 3' R:5'TCGCCAGCCCTCCCATGCCCATAA 3'	**60.2**
ACE (for expression)	F:5' -CCGAAATACGTGGAACTCATCAA-3' R:5'-CACGAGTCCCCTGCATCTACA- 3'	**58.8**
AGT ((M235T)	F:5'CCGTTTGTGCAGGGCCTGGCTCT 3'' R:5'CAGGGTGCTGTCCACACTGGACCCC 3'	**64.0**
RAGE (Gly82ser)	F:5' CACTGTTTAGGCCCTGCTTC 3' R:5' GGAATTCTTACGGTAGACACGG 3'	**62.1**
ALR(C-108T)	F:5' CCTTTCTGCCACGCGGGGCGCGGG 3' R:5' CATGGCTGCTGCGCTCCCCAG 3'	**66.0**
VEGF (I/D)	F:5'GCTGAGAGTGGGGCTGACTAGGTA 3' R:5'GTTTCTGACCTGGCTATTTCCAGG 3'	**58.7**
GAPDH (for expression)	F:5'-CCCATCACCATCTTCCAGGAG-3' R:5'-GTTGTCATGGATGACCTTGCC- 3'	**62.7**
B2M (for expression)	F:5' TAGCTGTGCTCGCGCTACT-3' R:5'TCTCTGCTGGATGACGTGAG-3'	**56.5**

Table 2: Demographic characteristics of study groups

Parameters	DN+DR(n=30)	DN-DR (n=30)	P-value
Gender (Male/Female)	15/15	17/13	0.796
Age(years)	51.90±11.53	49.9±10.55	0.486
Duration of diabetes (years)	9.61±6.37	6.81±5.60	0.071
Hypertension (Yes/No)	10/20	9/21	1.000
Duration of Hypertension (years)	1.01±2.13	2.40±5.26	0.188
Family history of diabetes (Yes/No)	12/18	11/19	1.000
Family history of hypertension (Yes/No)	6/24	4/26	0.731
Waist circumferences(cm)	89.13±10.98	96.48±9.39	**0.007**
BMI (kg/m^2)	24.68±4.56	26.76±3.56	0.053
SBP (mmHg)	145.70±23.64	139±19.04	0.258
DBP (mmHg)	82.53±7.46	83.37±11.70	0.744
Smoking (Yes/No)	4/26	4/26	1.000
Alcohol (Yes/No)	4/26	1/29	0.353
Other complications (Yes/No)	1/29	3/27	0.612

*All parameters have been mentioned as mean±1 SD

Table 3: Biochemical characteristics of study groups

Parameter	DN+DR(n=30)	DN-DR (n=30)	P-value
Blood urea (mg/dL)	35.56±14.99	30.75±16.34	0.240
Serum creatinine (mg/dl)	1.35±1.09	0.99±0.36	0.098
U.ACR$^{\#}$ (mg/g of creatinine)	2346.33 (72.29-17406.42)	1056.10 (54.24-9777.37)	0.243
eGFR (MDRD) (mL/min)	63.10±23.90	77.43±21.56	**0.018**

eGFR (EPI) (mL/min)	68.90±26.99	84.06±22.15	**0.021**
Serum Sodium ion (Na)(mmol/L)	140.30± 5.03	138.18± 2.14	0.243
Serum Potassium ion(K)(mEq/L)	4.98 ±0.95	4.55 ±0.52	**0.034**
Fasting plasma glucose (mg/dl)	257.97±122.60	221.13±76.02	0.167
Postprandial plasma glucose (mg/dl)	350.53±118.71	329.23±108.06	0.470
HbA1c (%)	10.02±1.94	9.92±2.39	0.855
Hb (%)	11.98±2.06	13.18±2.14	**0.031**
Total cholesterol (mg/dL)	188.67±61.66	195.41±61.51	0.673
Triglycerides (mg/dL)	199.41±125.79	202.08±95.28	0.926
HDLc (mg/dL)	41.22±10.20	36.08±7.73	**0.032**
LDLc (mg/dL)	121.63±41.72	128.08±51.88	0.598
VLDLc (mg/dL)	33.80±17.15	40.42±19.06	0.163

All parameters have been mentioned as mean± SD and #median (IQR)

Table 4: Distribution of genotypic frequencies between two groups

ACE I/D(n=60)		**DN+DR**	**DN-DR**	**p-value**
Genotype frequency	**II (wild)**	9	5	0.86
	ID	13	12	0.43
	DD (mutant)	8	13	0.42
AGT(M235T) (n=60)				
Genotype frequency	**MM (wild)**	19	10	0.50
	MT	6	9	0.33
	TT (mutant)	5	11	0.79
RAGE(Gly82Ser) (n= 60)				
Genotype frequency	**GG (wild)**	28	24	0.59
	GS	0	2	----
	SS (mutant)	2	4	0.28
ALR2 (C-106T) (n= 60)				
Genotype frequency	**CC (wild)**	21	24	0.09
	CT	6	6	0.64
	TT (mutant)	3	0	----
VEGF I/D (n=60)				
Genotype frequency	**II (wild)**	2	10	0.05
	ID	17	13	**0.03**
	DD (mutant)	11	7	**0.01**

*p <0.05 was considered statistically significant between two groups, DN= Diabetic nephropathy, DR= Diabetic retinopathy

Table 5: Relative mRNA expression (delta ct values) in ACE, AGT, RAGE, ALR and VEGF gene

Gene(n=30)	**DN+DR (mean delta ct)**	**DN-DR (mean delta ct)**	**P value**
ACE	2.96±1.10	2.33±1.31	0.051
AGT	3.35± 1.27	3.08 ±3,36	0.417
RAGE	3.07 ±1.21	2.78 ±1.34	0.391
ALR	2.02 ±1.55	1.35 ±1.30	0.076
VEGF	4.56 ± 1.29	1.49 ± 2.19	**≤ 0.001**

*p <0.05 was considered statistically significant between two groups, DN= Diabetic nephropathy, DR= Diabetic retinopathy

Table 6: Association of relative mRNA expression (delta ct values) and polymorphism of five genes between two groups

ACE I/D(n=60)		**DN+DR**	**DN-DR**	**p-value**
Genotype frequency	**II (wild)**	1.80±0.77	1.91±1.5	0.86
	ID	2.69±1.49	3.12±1.13	0.43
	DD (mutant)	2.37±1.43	3.23±0.67	0.42
AGT(M235T) (n=60)				
Genotype frequency	**MM (wild)**	3.23±1.35	2.91±.75	0.50
	MT	3.71±1.12	3.17±0.95	0.33
	TT (mutant)	3.41±1.28	3.15±2.02	0.79
RAGE(Gly82Ser) (n= 60)				
Genotype frequency	**GG (wild)**	3.09±1.24	2.91±1.26	0.59
	GS	0	3.96±0.49	----
	SS (mutant)	2.76±0.88	1.47±1.36	0.28
ALR2 (C-106T) (n= 60)				
Genotype frequency	**CC (wild)**	2.10±1.70	1.31±1.39	0.09
	CT	2.07±1.48	1.51±0.96	0.64
	TT (mutant)	1.54±0.92	0	-----
VEGF I/D (n=60)				
Genotype frequency	**II (wild)**	4.86±1.37	4.59±1.30	0.05
	ID	4.20±1.38	0.87±1.05	**0.03**
	DD (mutant)	5.08±1.05	0.62±1.02	**0.01**

*p <0.05 was considered statistically significant between two groups, DN= Diabetic nephropathy, DR= Diabetic retinopathy