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Original Article

Genetics Section

Genetic Polymorphism in Patients with Diabetic Nephropathy and Retinopathy: A Cross-sectional Study

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ABSTRACT

Introduction: The chronic persistence of diabetes leads to microvascular complications, such as Diabetic Nephropathy (DN) and Diabetic Retinopathy (DR). Both of these are progressive disorders involving pathological changes in capillaries. A few biochemical pathways have been suggested to link hyperglycaemia and microvascular complications. The probability of developing and progressing DN and DR is associated with the duration of diabetes. These complex disorders are strongly influenced by both genetics and environmental factors. Several candidate genes have been reported to be associated with DN and DR in different populations.

Aim: To determine the co-existence of DN and DR in relation to gene polymorphisms of Angiotensin-Converting Enzyme (ACE), Angiotensinogen (AGT), Receptor for Advanced Glycation End-products (RAGE), Aldose reductase (ALR2), and Vascular Endothelial Growth Factor (VEGF).

Materials and Methods: The present DN and DR cross-sectional study was conducted at the Department of Endocrinology, University College of Medical Sciences, University of Delhi and GTB, New Delhi, India, and the Discipline of Biochemistry, Indira Gandhi National Open University, New Delhi, India. The study was conducted from October 2019 to August 2022. All the participants under uniform diabetes management were divided into two groups (100 in each group): Group 1 included Type-II diabetic patients with DN and DR, and Group 2 comprised Type-II diabetic patients with DN only. Polymorphisms in all genes were determined using Polymerase Chain Reaction (PCR), followed by digestion with restriction enzymes and visualisation through an ultraviolet transilluminator. The analysis of biochemical parameters and association of gene polymorphisms was performed using Statistical Package for Social Sciences (SPSS) version 26.0 software.

Results: There were 57 males and 43 females in the DN+DR group, and 51 males and 49 females in the DN-DR group. The mean age of study subjects in the DN+DR group was 52.60±9.08 years, compared to the DN-DR group (47.33±10.68 years). The DN+DR group had a significantly higher mean duration of diabetes (11.78±6.86 vs. 5.13±4.78, p≤0.001) and a significantly lower mean waist circumference (91.30±13.99 vs. 95.11±9.95 cm, p≤0.02). The DN+DR group also had significantly higher urea (34.34±16.32 vs. 26.43±12.34 cm, p≤0.001), creatinine (1.34±0.90 vs. 0.89±0.25 cm, p≤0.03) and significantly lower estimated Glomerular Filtration Rate (eGFR) levels compared to the DN-DR group. The distribution of genotypes of ALR2 (p≤0.04) and VEGF genes (p≤0.001) showed a significant difference between both DN+DR and DN-DR groups. The frequency of the D allele of the VEGF gene (p≤0.02) (OR=1.94, 95% Confidence Interval (CI)=1.10-3.40) was higher in the DN+DR group. The DN+DR group also had a significantly lower frequency of the CT+TT dominant model of the ALR2 gene (p≤0.04) as well as an increased frequency of the ID+DD dominant model of the VEGF gene (p≤0.002). No significant differences were found in genotypic as well as allelic frequencies of ACE, AGT, and RAGE gene polymorphisms between the two groups.

Conclusion: The D allele of the VEGF (I/D) gene polymorphism is significantly associated with DR in patients with DN. It can be concluded that the VEGF gene plays an important role in the development of retinopathy in DN patients.

Keywords: Diabetes, Polymorphism, Vascular endothelial growth factor

INTRODUCTION

Diabetes mellitus, a hyperglycaemic condition, is caused by an abnormality in insulin secretion and/or function. According to the International Diabetes Federation (IDF) Atlas (10th edition), in 2021 approximately 537 million individuals aged 20-79 years suffered from diabetes, accounting for one death every five seconds. The number of diabetic patients is projected to rise to 643 million cases by 2030 and 783 million by 2045 [1]. In India, the prevalence of this rapidly growing health challenge and the potential for a diabetic epidemic are high across the lower- and middle-income states [2]. It is predicted that the number of diabetic patients in India will increase to 69.9 million cases by 2025 [2,3].

Microvascular complications of diabetes are the leading causes of early mortality and morbidity in diabetes, representing the leading cause of blindness and End-Stage Renal Disease (ESRD) [4,5]. DN patients present with persistent microalbuminuria (≥30-299 mg/g of creatinine), which further progresses to macroalbuminuria (≥300 mg/g of creatinine) [4]. It is now reported that only one-third of DN patients have DR [6,7]. DR is characterised by aneurysms, neovascularisation with poorly formed weak vessels, vascular rupture, and bleeding in the retina. Several factors, such as family history of diabetes or hypertension, poor glycaemic control, and duration of diabetes, may predispose to the development of both complications of diabetes. However, not all DN patients tend to be associated with retinopathy. Therefore, common underlying hereditary factors could play a substantial role in determining the association of DR and DN. There is a correlation between chronic diabetic conditions and genetic polymorphisms of genes such as the Renin Angiotensin Aldosterone System (RAAS), RAGE, VEGF, and ALR2 [8].

Two polymorphisms of the RAAS gene have been studied: ACE I/D and AGT-M235T polymorphism. It has been observed that the D allele and T allele are associated with DN, respectively [9]. RAGE, a pattern recognition receptor present on the cell surface, is involved in various pathophysiological activities and is responsible for the initiation of signaling cascades. It has been observed that cytokine

secretion due to the activation of RAGE enhances inflammation and permeability of the endothelial membrane, thereby exacerbating diabetic complications [10]. ALR2 activation in the polyol pathway leads to the conversion of glucose to sorbitol. In a hyperglycaemic condition, sorbitol accumulates inside the cell, causing osmotic stress. The overproduction of NADH leads to oxidative stress in the cell. Such conditions worsen diabetic complications such as DN and DR [11].

The VEGF is a cytokine important for angiogenesis and cell permeability. VEGF polymorphism worsens glomerular injury, endothelial dysfunction, and raised blood vessel permeability, as observed in both DN and retinopathy [12]. To date, there are very few reports regarding the co-existence of DN and DR in Type 2 Diabetic Patients (T2DM) [6,13]. This current study was planned as a cross-sectional study to ascertain whether common underlying genetic factors have a role in the association of retinopathy and nephropathy in patients with diabetes. The present study focuses on investigating the differential distribution of genotypes and alleles of five genes: ACE, AGT, RAGE, ALR2, and VEGF individually with the occurrence of DR among T2DM patients with DN.

MATERIALS AND METHODS

The present cross-sectional study was carried out in the outpatient clinics of the Centre for Diabetes, Endocrinology, and Metabolism (DEM) of University College of Medical Sciences (UCMS) and GTB Hospital, Delhi, India. The current study proposal was approved by the Institutional Ethics Committee-Human Research (IEC-HR) of UCMS, Delhi (Ref.No., IEC-HR/2019/38/4R). The duration of the study was from October 2019 to August 2022. A total of 200 type 2 diabetic patients diagnosed with DN were enrolled in the hospital for the study.

Inclusion criteria: Subjects with T2DM aged between 20-70 years with evidence of persistent microalbuminuria (30-300 mg/g of creatinine) or macroalbuminuria (>300 mg/g of creatinine), with or without DR, were included.

Exclusion criteria: Patients on Non Steroidal Anti-Inflammatory Drugs (NSAIDs), nephrotoxic drugs, or with any urinary or systemic infection were excluded from the study.

Study Procedure

The study subjects were divided into two groups. Group 1 (n=100) consisted of patients with DN with retinopathy (DN+DR), whereas Group 2 (n=100) included patients with DN without retinopathy (DN-DR). All patients underwent direct fundoscopic examination by an ophthalmologist to detect DR. Diabetes mellitus in the study subjects was diagnosed based on the American Diabetes Association guidelines (ADA) 2017 [14].

Sample collection and genomic Deoxyribonucleic Acid (DNA) extraction: For genomic Deoxyribonucleic Acid (DNA) extraction, peripheral venous blood (5 mL) from the studied patients was collected in Ethylene Diamine Tetra Acetic Acid (EDTA)-coated tubes. DNA was isolated from whole blood using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions and stored at -80°C until further molecular analysis [15]. The quality and quantity assessment of the extracted DNA was performed using NanoDrop-2000, Thermo Scientific. Absorbance at 260/280 nm was measured in the Multidisciplinary Research Unit (MRU), and samples with an optical density close to 1.8 were considered pure DNA and selected for further experiments [16].

Primer designing: The complete domain sequence of all selected genes was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed). Primers for genotyping analysis were designed using the online Primer3 software (http://primer3.ut.ee/). The designed sequences were then analysed for potential hairpin formation and self-complementarity using the online OligoCalc tool (http://www.basic.northwestern.edu/biotools/oligocalc.html). The primers used in the present study are listed in [Table/Fig-1].

S. No.	Gene	Primer sequence	Annealing temperature (°C)
1	ACE gene (I/D) (Flanking pair)	F: 5'CTGGAGACCACTCCCATCCTTTCT 3' R: 5'GATGTGGCCATCACATTCGTCAGAT 3'	62.30
2	ACE (I/D) (Insertion-specific pair)	F: 5'TGGGACCACAGCGCCCGCCACTAC 3' R: 5'TCGCCAGCCCTCCCATGCCCATAA 3'	60.20
3	AGT gene (M235T)	F: 5'CCGTTTGTGCAGGGCCTGGCTCT 3' R: 5'CAGGGTGCTGTCCACACTGGACCCC 3'	65.70
4	RAGE (Gly82Ser)	F: 5'CACTGTTTAGGCCCTGCTTC 3' R: 5'GGAATTCTTACGGTAGACACGG 3'	56.00
5	ALR2 gene (C-106T)	F: 5'CCTTTCTGCCACGCGGGGCGCGGG 3' R: 5'CATGGCTGCTGCGCTCCCCAG 3'	66.00
6	VEGF gene (I/D)	F: 5'GCTGAGAGTGGGGCTGACTAGGTA 3' R: 5'GTTTCTGACCTGGCTATTTCCAGG 3'.	58.70

[Table/Fig-1]: Specific primers used for ACE, AGT, RAGE, ALR2 and VEGF gene for genotyping.
F: Forward primer; R: Reverse primer

Determination of genotypes: The polymorphism in genes ACE I/D, AGT M235T, RAGE (gly82ser), ALR2 (C-108T), and VEGF I/D was analysed by PCR amplification of the extracted DNA followed by restriction endonuclease digestion. After initial denaturation for five minutes at 95°C, 35 amplification cycles were performed with a PCR machine (Bio-Rad T100TM Thermal Cycler) using a preset programme {initial denaturation for 30 seconds at 95°C followed by annealing for 60 seconds at 62.3ºC for ACE I/D, 65.7ºC 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]. 25 µL PCR reaction mixture included 100 ng of genomic DNA and extension for one PCR reaction mixture (25 µL) included 100 ng of genomic DNA and 10 pmol of primer. The PCR master mix contained 0.2 µL of 5 U Taq DNA polymerase, 0.5 µL of 200 µL of each dNTPs, 1X PCR buffer, and 3 mM of MgCl2. The volume of the master mix was made up to 22 µL with nuclease-free water. Each reaction contained 22 µL of the master mix and 3 µL of DNA (50-150 ng/µL) solution. The amplification of the ACE gene was performed in a two-step process using a pair of flanking primers that distinguish insertion-specific sequences. The PCR product was then digested with restriction enzymes for AGT (Tth111I) (NEB), ALR2 (BfaI) (NEB), and RAGE (AluI) (NEB) to identify the M/T, C/T, and G/S polymorphisms, respectively, at 37°C for 16 hours. The digested DNA fragments were electrophoresed on a 2% agarose gel and visualised using a UV transilluminator [17,18].

STATISTICAL ANALYSIS

The SPSS version 26.0 software was used for statistical analysis. Differences between the two groups were analysed using an independent Student's t-test. The significance of the demographic data between both groups was determined using the Chi-square test. The association between polymorphisms in five genes and susceptibility to DN and DR was determined using odds ratios with a 95% CI under two genetic models: dominant and recessive. A p-value of ≤0.05 was considered statistically significant. The distribution of polymorphisms between the two groups was assessed using the Hardy-Weinberg equation and Chi-square test.

RESULTS

Demographic and biochemical characteristics of the study population: A total of 200 participants were enrolled in the study,

divided into two groups: DN patients with retinopathy (DN+DR) and DN patients without retinopathy (DN-DR), with 100 patients in each group. The demographic parameters of the study subjects in both groups are shown in [Table/Fig-2]. The DN+DR group had 57 males and 43 females, while the DN-DR group had 51 males and 49 females. The mean age of the study subjects was higher in the DN+DR group (52.60±9.08 years) compared to the DN-DR group (47.33±10.68 years). The DN+DR group had a significantly longer mean duration of diabetes (11.78±6.86 vs. 5.13±4.78 years, p≤0.001) and significantly lower mean waist circumference (91.30±13.99 vs. 95.11±9.95 cm, p≤0.02). Systolic Blood Pressure (SBP) was significantly higher (143.85±21.92 vs. 137.0±19.73 mmHg, p≤0.02) and Diastolic Blood Pressure (DBP) (82.30±9.75 vs. 85.76±11.71 mmHg, p≤0.02) was significantly lower in the DN+DR group compared to the DN-DR group.

Parameters	DN+DR (n=100)	DN-DR (n=100)	p-value
Gender (Male/Female)	57/43	51/49	0.395
Age (Years)	52.60±9.08	47.33±10.68	**<0.001**
Duration of diabetes (Years)	11.78±6.86	5.13±4.78	**<0.001**
Hypertension (Yes/No)	53/47	64/36	0.114
Duration of Hypertension (Years)	1.84±4.32	1.83±4.3	0.578
Family history of diabetes (Yes/No)	35/65	41/59	0.382
Family history of hypertension (Yes/No)	17/83	17/83	1.000
Waist circumferences (cm)	91.30±13.99	95.11±9.95	**0.028**
BMI (kg/m^2)	27.33±7.14	27.34±4.53	0.997
SBP (mmHg)	143.85±21.92	137.0±19.73	**0.021**
DBP (mmHg)	82.30±9.75	85.76±11.71	**0.021**
Smoking (Yes/No)	11/89	11/89	1.000
Alcohol (Yes/No)	11/89	9/91	0.637
Other complications (Yes/No)	7/93	8/92	0.788

[Table/Fig-2]: Demographic characteristics of study groups (N=200).
*All parameters have been mentioned as mean±1 SD, DN: Diabetic nephropathy; DR: Diabetic retinopathy

Biochemical parameters among the two study groups are shown in [Table/Fig-3]. The DN+DR group had significantly higher levels of urea (34.34±16.32 vs. 26.43±12.34 mg/dL, p≤0.001), creatinine (1.34±0.90 vs. 0.89±0.25 mg/dL, p≤0.03), median UACR (394.92 v/s 178.42 mg/g, p<0.002), mean serum potassium (5.29±3.86 vs. 4.47±0.54 mmol/L, p<0.03), and significantly lower eGFR (MDRD) (estimated glomerular filtration rate) (modified diet in renal disease) (61.05±21.05 v/s 85.22±21.35 mL/min/1.73 m^2, p≤0.001), eGFR (EPI) (estimated glomerular filtration rate) (epidemiology collaboration) (66.42±23.24 v/s. 91.75±19.48 mL/min/1.73 m^2, p≤0.001), and mean haemoglobin levels (11.96±1.86 vs. 12.91±1.86 g/dL, p≤0.001) compared to the DN-DR group.

Parameters	DN+DR (n=100)	DN-DR (n=100)	p-value
Blood urea (5-20 mg/dL)	34.34±16.32	26.43±12.34	**<0.001**
Serum creatinine M-0.7-1.3 mg/dL F-0.5-1.2 mg/dL	1.34±0.90	0.89±0.25	**<0.001**
U.ACR# Microalbuminuria- 30-300 mg/g of creatinine Macroalbuminuria- >300 mg/g of creatinine	394.92 (37.80-17406)	178.42 (32.96-9777.37)	**0.002**
eGFR (MDRD) (mL/min)	61.05±21.05	85.22±21.35	**<0.001**
eGFR (EPI) (mL/min)	66.42±23.24	91.75±19.48	**<0.001**
Serum Sodium ion (Na) (136-145 mmol/L)	138.31±13.58	137.73±6.32	0.221
Serum Potassium ion (K) (3.5-5.2 mEq/L)	5.29±3.86	4.47±0.54	**0.038**
Fasting plasma glucose (60-99 mg/dL)	225.64±101.71	222.85±72.02	0.838
Postprandial plasma glucose (80-120 mg/dL)	306.94±101.78	320.16±101.62	0.368
HbA1c (4-5.6%)	10.20±2.40	9.79±2.12	0.203
Hb (M-14-18(%), F-12-16(%))	11.96±1.86	12.91±1.86	**<0.001**
Total cholesterol (125-200 mg/dL)	186.31±54.15	189.66±62.03	0.685
Triglycerides (<150 mg/dL)	196.37±267.97	183.52±125.57	0.665
HDLc (35-80 mg/dL)	41.27±26.23	38.58±9.59	0.525
LDLc (<100 mg/dL)	116.51±41.39	120.55±42.08	0.495
VLDLc (2-30 mg/dL)	38.13±53.87	36.70±25.11	0.811

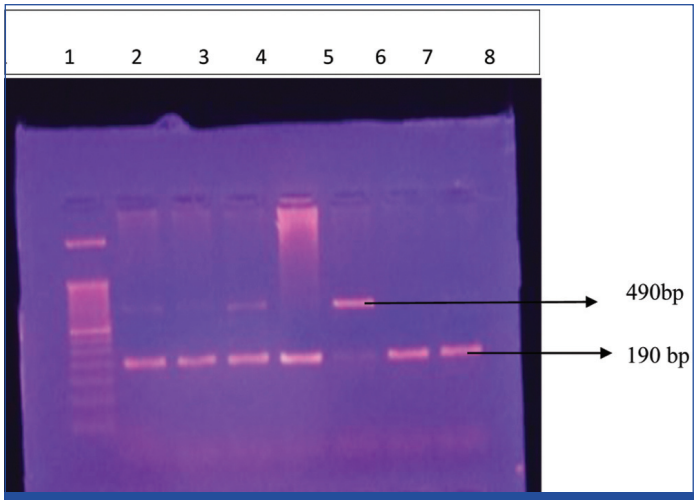
[Table/Fig-3]: Comparison of biochemical parameters of study groups.
*All parameters have been mentioned as mean±SD and #median (IQR), DN: Diabetic nephropathy; DR: Diabetic retinopathy

Genotypic frequency distribution of gene polymorphisms: The genotype distribution and allele frequencies of ACE, AGT, RAGE, ALR2, and VEGF genes are shown in [Table/Fig-4] and [Table/Fig-5-9]. There were significant differences in the distribution of genotypes of VEGF (I/D) (p≤0.001) and C-108T of ALR2 (p<0.04) genes between the two groups. However, there were no significant differences in the genotypic and allelic frequencies of ACE {Insertion/Deletion (I/D)}, AGT (M235T), and RAGE (gly82ser) gene polymorphisms between the two groups.

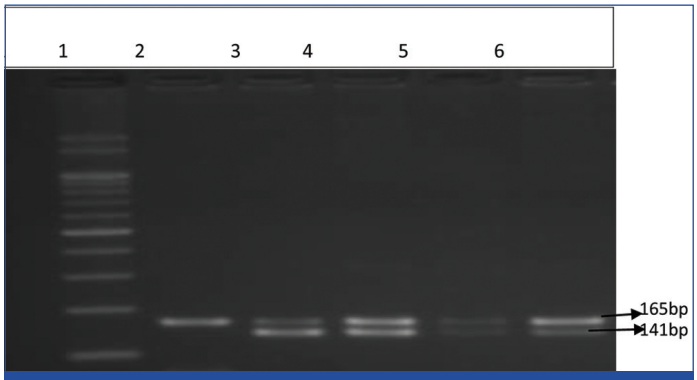
ACE (I/D) (N=200)		DN+DR (%)	DN-DR (%)	p-value
Genotype frequency	II (wild)	29	22	0.104
	ID	26	40	
	DD (mutant)	45	38	
Allele frequency	I	42	42	1.000
	D	58	58	
AGT (M235T)				
Genotype frequency	MM (wild)	46	54	0.301
	MT	35	25	
	TT (mutant)	19	21	
Allele frequency	M	63.5	66.5	0.652
	T	36.5	33.5	
RAGE (Gly82Ser)				
Genotype frequency	GG (wild)	91	88	0.781
	GS	4	5	
	SS (mutant)	5	7	
Allele frequency	G	93	90.5	0.461
	S	7	9.5	
ALR2 (C-106T)				
Genotype frequency	CC (wild)	84	72	**0.04***
	CT	9	22	
	TT (mutant)	7	6	
Allele frequency	C	88.5	83	0.302
	T	11.5	17	
VEGFI/D				
Genotype frequency	II (wild)	20	40	**<0.001***
	ID	40	33	
	DD (mutant)	40	27	
Allele frequency	I	40	56	**0.02***
	D	60	43.5	

[Table/Fig-4]: Distribution of genotypic and allelic frequencies between two groups.
*p≤0.05 was considered statistically significant between both groups, DN: Diabetic nephropathy; DR: Diabetic retinopathy

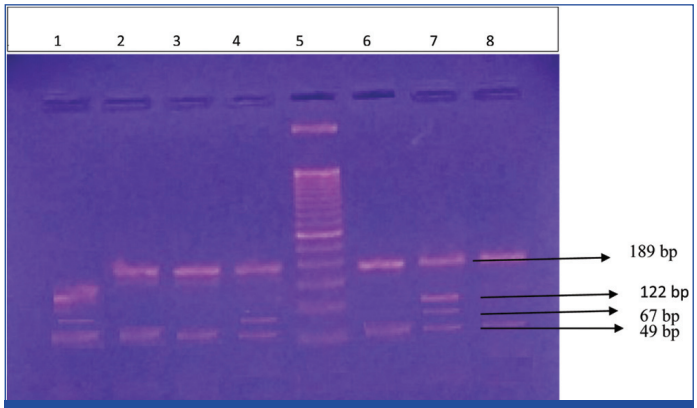
Analysis of five gene polymorphisms in two groups: The genotypic frequencies of ACE, AGT, RAGE, ALR2, and VEGF gene polymorphisms were evaluated using odds ratios with 95% CIs



[Table/Fig-5]: ACE I/D polymorphism analysis by allele-specific PCR method. Lane 1 is 50 bp DNA ladder. Lanes 2 and 5 are showing homozygous wild type genotype (II). Lanes 4, 7 and 8 are showing heterozygous type genotype (ID). Lanes 3 and 6 are showing homozygous mutant genotype (DD).



[Table/Fig-6]: AGT(M235T) polymorphism analysis by allele-specific PCR method. Lane 1 is 100 bp DNA ladder. Lane 2 is showing homozygous wild type genotype (MM). Lanes 3, 4, 5 and 6 are showing heterozygous type genotype (MT).

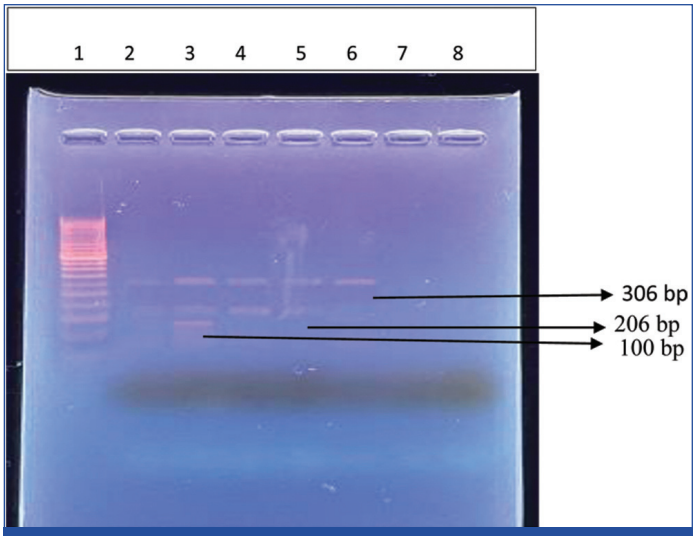


[Table/Fig-7]: RAGE (gly82ser) polymorphism analysis by allele-specific PCR method. Lane 5 is 50 bp DNA ladder. Lanes 2, 3, 6, and 8 are showing homozygous wildtype genotype (GG). Lanes 1 and 4 are showing homozygous mutant genotype (SS). Lane 7 is showing heterozygous type genotype (GS).

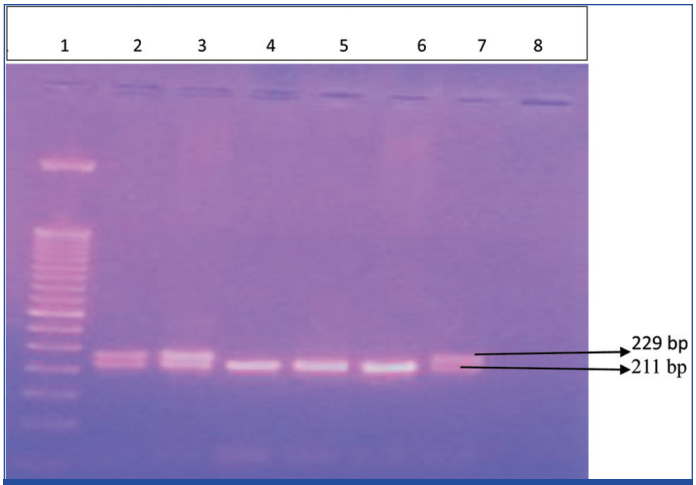
[Table/Fig-10]. A positive association was observed between VEGF I/D polymorphism and retinopathy in DN patients, while a negative association was seen with ALR2 C108-T polymorphism. The D allele of VEGF gene was significantly associated with the DN+DR group (OR=2.66, 95% CI=1.41-5.02, p<0.002) in the dominant model, suggesting that it is a risk allele for diabetes nephropathy with retinopathy. On the other hand, the T allele of ALR2 C-108T gene polymorphism was significantly associated with a decreased risk of diabetes nephropathy with retinopathy (OR=0.49, 95% CI=0.246-0.977, p<0.04), indicating that it is a protective allele.

DISCUSSION

It is generally observed that 80%-90% of DN patients have retinopathy due to type 2 diabetes [19]. DN and DR affect 40%



[Table/Fig-8]: ALR2 (C-108T) polymorphism analysis by allele-specific PCR method. Lane 1 is 50 bp DNA ladder. Lanes 2, 4 and 5 are showing homozygous wildtype genotype (CC). Lane 6 is showing homozygous mutant genotype (SS). Lane 3 is showing heterozygous type genotype (CT).



[Table/Fig-9]: 18bp fragment (I/D) polymorphism at -2549 position of VEGF gene analysis by allele-specific PCR method. Lane 1 is 50 bp DNA ladder. Lanes 2, 3 and 7 are showing heterozygous mutant type genotype (ID). Lanes 4, 5 and 6 are showing homozygous mutant genotype (DD).

and 27% of global chronic diabetic patients, respectively [20,21]. Numerous reports have highlighted the association of Single Nucleotide Polymorphisms (SNPs) in genes with the risk of DR [8,22,23]. Several candidate genes have been explored for variations and their association with DR, but a comprehensive understanding of the pathogenic mechanisms underlying DR is still needed. In the present study, the authors observed a significant association between retinopathy and the duration of diabetes. This finding is consistent with several other studies [6,24], indicating that the prevalence of retinopathy increases with the duration of diabetes. The authors also found a significant and positive association of the DN+DR group with decreased renal function markers such as blood urea, serum creatinine, Urinary Albumin-to-Creatinine Ratio (UACR), estimated Glomerular Filtration Rate (eGFR), and serum potassium levels compared to the DN-DR group. These results align with previous studies [25-27], which have shown that the occurrence of retinopathy is related to a decline in renal function parameters and an increase in serum creatinine levels.

During genetic analyses, it is important to carefully select patients, and single-centre analyses are preferable. Such patient selection can reveal an even stronger effect of a suspected allele or genotype if the dominant allele or genotype is associated with a specific target disorder. In the present study, the authors analysed patients from a single hospital, ensuring uniform management of diabetes. The two groups, DN+DR and DN-DR, were based on their age,

Type of model	Polymorphism	Genotype	DN+DR	DN-DR	OR	95% CI	χ^2	p-value
Dominant model	ACE	ID+DD	71	78	0.691	0.364-1.30	1.29	0.256
		II	29	22				
	AGT	MT+TT	53	46	1.37	0.790-2.40	1.20	0.288
		MM	46	54				
	RAGE	GS+SS	9	12	0.725	0.291-1.80	0.479	0.479
		GG	91	88				
	ALR	CT+TT	16	28	0.490	0.246-0.977	4.19	0.041
		CC	84	72				
	VEGF	ID+DD	80	60	2.66	1.41-5.02	9.52	0.002
		II	20	40				
Recessive model	ACE	DD	45	38	0.749	0.426-1.31	1.00	0.315
		II+ID	55	62				
	AGT	TT	19	21	1.13	0.566-2.26	0.125	0.724
		MM+MT	81	79				
	RAGE	SS	5	7	1.43	0.438-4.66	0.355	0.552
		GG+GS	95	93				
	ALR	TT	7	6	0.848	0.275-2.61	0.08	0.774
		CC+ CT	93	94				
	VEGF	DD	40	27	0.555	0.306-1.00	3.79	0.052
		II+ ID	60	73				

[Table/Fig-10]: Analysis of polymorphisms of five genes between two groups.
*p≤0.05 was considered statistically significant between both groups, DN: Diabetic nephropathy; DR: Diabetic retinopathy

onset of diabetes and duration of diabetes. However, since the study was hospital-based, it was important to consider potential biases.

The present study results showed a significant association between the I/D polymorphism of the VEGF gene and DR risk in DN patients, with the DD genotype and D allele frequencies being notably correlated (p≤0.001 and p≤0.02, respectively). These findings align with a study by Khan SZ et al., (2020) in the Pakistani population, suggesting that the DD genotype and D allele of the VEGF gene may be related to diabetic complications [22]. Other studies have also reported associations between VEGF gene polymorphism and retinopathy in different populations [24].

Referral bias cannot be excluded, even though patients can visit the centre without a referral. The present results revealed no associations between ACE, RAGE, and AGT gene polymorphisms and DN in diabetic patients. These findings are consistent with a previous study by Miura J et al., (1999), suggesting that genetic differences in these polymorphisms are unlikely to play a significant role in predisposition to Diabetic Retinopathy (DR) in DN patients [28]. Discrepancies in other studies may be attributed to ethnic differences and lifestyle factors.

The VEGF is an essential factor in angiogenesis and vascular permeability of endothelial cells. In diabetes, VEGF activation occurs due to hypoxia and high blood glucose levels, leading to the destruction of the Blood-Retinal Barrier (BRB) and the development of diabetic macular edema and neovascularisation [29]. A study done by Amle D et al., reported associations between VEGF gene polymorphism and diabetic nephropathy in T2DM population of North India [30]. Furthermore, VEGF gene polymorphism results demonstrated a strong association between the DD genotype and the D allele with retinopathy in DN patients, with an odds ratio of 2.66 (1.41-5.02) in the dominant model, indicating that the D allele may be a potential risk factor for retinopathy progression in DN patients. Gala-Błądińska A et al., (2019) conducted a study confirming the involvement of the D allele in VEGF-related complications [31].

A gene polymorphism with an odds ratio of 1.31 (p≤0.033) has been found to increase the risk of Diabetic Retinopathy (DR) among all diabetic vascular complications in the Caucasian population [31]. In contrast to the aforementioned study, research has shown a higher odds ratio, a wider range of confidence intervals, and a significant difference with a higher frequency of the ID+DD genotype in the DN+DR group, indicating an increased risk of retinopathy in DN subjects. ALR2, the first enzyme of the polyol pathway, is considered a key player in linking hyperglycaemia to diabetes complications. It converts glucose into sorbitol, leading to sorbitol accumulation and osmotic stress in the presence of high blood glucose. The ALR2 gene, located at position 7q35.12, contains a common polymorphism in the promoter region at nucleotide C/T at position 106, which has been studied for its association with the risk of DR. However, studies on the contribution of ALR2 polymorphism to DR have yielded conflicting results, with some supporting the association while others do not.

In the present study, the TT genotype of the C-106T polymorphism of the ALR2 gene appeared to be protective against DR, showing a significant difference in genotypic frequencies but not in allelic frequencies. The present study findings align with a study by Wang Y et al., suggesting that ALR2 gene polymorphism may be linked to microvascular complications of diabetes [32]. However, the observations by Gosek K et al., (2005) contradict the present study, reporting C-106T polymorphism as a risk factor for DN development in type 2 diabetic subjects with poor glycaemic control [33]. Olmos P et al., also stated an association between this ALR2 gene polymorphism and the development of DR in the Chilean population [34]. The conflicting outcomes may be attributed to differences in ethnicity.

The present study results regarding the ALR2 gene polymorphism demonstrated an association between the T allele and protection against DR in DN patients, with an odds ratio of 0.49 (0.246-0.977) in the dominant model, suggesting that the T allele may act as a protective allele for DR. DN patients in the present study who had this allele were protected from DR and did not experience DN for many years. These findings are supported by Sivenius K et al., who observed a similar association between the T allele of the ALR2 gene polymorphism and albuminuria in the Finnish

population with type 2 diabetes mellitus [35]. Another study by Cui W et al., reported a correlation between the C-106T polymorphism of the ALR2 gene and the susceptibility to DN, rather than its progression [36]. The present study results were consistent with these observations. When comparing the T allele with the C allele, the C allele, which exhibits high mRNA expression levels, may result in inefficient conversion of glucose into sorbitol by ALR, leading to sorbitol accumulation and subsequent retinopathy due to osmotic stress.

However, Deng Y et al., reported that the T allele of the ALR2 gene is not significantly associated with DR in Chinese patients with T2DM, both with and without retinopathy [37]. The contradiction in results can be attributed to ethnic and regional differences, small sample sizes, different methods of sample recruitment, and variations in the mechanisms underlying the development and progression of DR. The authors were the first to report that the T allele of the ALR2 C-106T polymorphism is a protective allele for DR in patients with DN among the North Indian population. To validate the role of VEGF and ALR2 gene polymorphisms in DN with and without retinopathy, further studies with larger sample sizes are required.

Limitation(s)

A limitation of the present study was the small sample size in the study groups. In genetic studies, small sample populations tend to lose genetic diversity more rapidly than large sample populations due to genetic drift. This is because certain gene forms can be lost by chance, and this is more likely to occur when the population size is small. Case-control designs that include healthy controls could be more suitable for identifying the significant role of these genes.

CONCLUSION(S)

In the present study, the authors found that the D allele of the VEGF (I/D) polymorphism may be a risk factor, while the T allele of the ALR2 (C-108T) polymorphism may have a protective role in relation to DR in DN patients. For future studies with a solid clinical impact, there is a dire need to further establish and elucidate the role of candidate genes and their respective polymorphisms with a larger sample size in patients with DN and retinopathy.

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