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Association Of VEGF And Microrna-221 Expression With MTHFR Gene Polymorphism; A Molecular Insight Into Angiogenic And Genetic Regulatory Pathways

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Abstract
Diabetic retinopathy (DR), a prominent microvascular consequence of type 2 diabetes, is influenced by a wide range of variables, both hereditary and physiological. One hundred fifty samples of blood were collected from human subjects for the purpose of this study. Participants were divided into three equal groups: diabetics with retinopathy (DR), diabetics without retinopathy (DM), and healthy adults without diabetes (control). A study was conducted on the genetics of two MTHFR single-nucleotide polymorphisms (rs1801133 and rs1801131), along with the expression analysis of the VEFG, and miR-221 genes. The AA genotype at rs1801133 is associated with a heightened risk of diabetic retinopathy ($p = 0.011$), while the TT genotype at rs1801131 correlates with increased homocysteine levels. This was identified through the analysis of genetic polymorphisms in the MTHFR gene. The DR group exhibited a noteworthy elevation in MTHFR gene expression, which was correlated with a significant increase in VEGF expression. This indicated the activation of augmented angiogenic pathways linked to retinal damage. It is possible that the DR group is responsible for the highest levels of gene expression of microRNA-221 (miR-221), which suggests that it plays a role in inflammatory responses and cell-mediated immunity in the context of DR. The study found physiologic and genetic causes of diabetic retinopathy. Gene expression analysis, genetic polymorphisms, and conventional biomarkers enhance early diagnostic and prognostic tools. These findings emphasize to the need for multi-level research and personalized medicine in diabetic complications to better comprehend pathogenic mechanisms and develop effective treatments. The study's findings shed light on one of diabetes' most serious retinal damage complications and aid in treatment.

Keywords: Diabetic retinopathy, MTHFR gene, folate metabolism, gene expression, metabolic pathways, RT-PCR.

INTRODUCTION

1–3 Diabetic retinopathy (DR) is an incapacitating microvascular complication of diabetes mellitus, which affects millions of people globally and represents a heavy public health problem. It is clinically

characterized by the progressive destruction of retinal vasculature, ultimately resulting in vision loss and blindness in its untreated stage. Despite progress in the treatment modalities of Diabetic retinopathy, its pathogenesis is still not sufficiently understood and environmental and genetic factors take center stage. (Campochiaro and Akhlaq, 2021; Cao et al., 2024).

Recently, investigating the role of genetics in DR is increasing with several studies published focusing on genetic polymorphisms in association with DR severity and susceptibility. Genetic features of diabetic retinopathy MTHFR gene, VEGF gene, and microRNA-221 (miR-221) are involved in disease pathogenesis and their importance is increasing for the development of diseases (Sienkiewicz-Szłapka et al., 2023; Smoleński et al., 2021). In this study, MTHFR and VEGF genes as well as miR-221 were the main focus under diabetic retinopathy in conjunction with pathogenesis of many chronic micro -vascular diseases.

The methylenetetrahydrofolate reductase enzyme, which is encoded by the MTHFR gene on chromosome 1p36.3, is necessary for folate metabolism and the control of homocysteine (Moin et al., 2021). The MTHFR gene contains two common functional polymorphisms, rs1801133 and rs1801131, which have been thoroughly investigated in relation to DR. The pathophysiology of DR is linked to these polymorphisms because they have been linked to changed activity of enzyme, which causes disturbance in metabolism of homocysteine, endothelial dysfunction, and oxidative stress (Jiang et al., 2023). Further research into MTHFR polymorphism's role in disease progression is essential because results about their association with DR risk have been inconsistent across studies.

The involvement of VEGF gene, along with MTHFR, in the pathogenic mechanism of diabetic retinopathy has been reported. This factor is a potent angiogenic mediator that controls the plasticity of neovascularization and vessel permeability, both of which are disturbed during diabetic retinopathy (DR). VEGF levels have been shown to be increased in the vitreous and into the retina of diabetes and diabetic retinopathy, however, there is little documented impact of VEGF inhibitors on ocular complications (Zhang et al. 2023). Influencing retinal vascular homeostasis and participating in the pathological angiogenesis seen in DR(Huo et al., 2024).

Emerging literature tends to prove that miRNAs, such as miR-221, are responsible for DR development. MicroRNAs (miRNAs), small/ non-coding RNAs which regulate gene expression at the post-transcriptional level, are essential in angiogenesis, inflammation and apoptosis. MiR-221 is misexpressed in the retina and vitreous of DR patients, appearning to contribute to disease pathogenesis (Zhao et al., 2022). or miR-221, may mediate abnormal neovascularization of DR and contribute to vascular impairment by regulating angiogenic signaling pathways such as VEGF (vascular endothelial growth factor) and eNOS (endothelial nitric oxide synthase).
(Wang et al., 2022).

The polymorphisms in the VEGF genes and collectively with miRNA-221, are expected to have a substantial impact on the susceptibility as well as severity of DR and focusing our attention toward genetic factors for DR susceptibility and progression is essential to increase of understanding about its basic pathophysiological mechanisms. The results of this study would be important, as they may help in the development of new therapeutic targets by improving the ways to handle and prevent DR In short, investigators aim to deliver a solid evidence-based foundation for these genetic factors and their roles in diabetic retinopathy.

MATERIALS AND METHODS

Subjects:

In the present study, 50 Iraqi patients with DR who were clinically diagnosed at Al-Kadhimiya Medical in Baghdad were included, in addition to 50 apparently healthy individuals as negative control group and 50 diabetic patients without retinopathy as positive control group ,who were matched with DR patients in age and sex. All subjects have been informed about the aim of this study and asked to fill in the questionnaire form.

Blood sampling :

A blood sample (3 mL) was drawn from each participant. The collected blood was split into two parts: 2 mL went into an EDTA tube and 1 mL of whole blood was collected in a trizol tube to check gene expression.(250 µl) was added to (750 µl) of trizol for RNA the estimation of gene expression,The EDTA-anticoagulated blood was used for genetic analysis. The samples were stored at -20ºC until further processing.The expression levels of VEGF gene and microRNA-221 were calculated using the ΔCt method where the fold change values were determined for the DR patient group compared with the negative control group.

Total RNA extraction:

RNA was extracted from blood samples according to the protocol of TRIzol™ Reagent using)ELK Biotechnology, Chine, EP013) according to the manufacturer instructions (Abdulhameed and mohammed, 2022). Quantus fluorometer was used to detect the concentration of extracted RNA in order to detect the quality of samples for downstream applications. For 1 µl of RNA, 199 µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, RNA concentration was detected.

cDNA synthesis:

A total of 2 µgRNA was used for reverse transcription (RT) with the TransCriptor First-Strand cDNA Synthesis Super mix according to the manufacturer's instructions (Synthol /Russia).

Synthesis of primers:

In this study, single nucleotide polymorphisms (SNPs) within the MTHFR gene were genotyped utilizing the TaqMan 5′-allele discrimination method, founded on the principles of real-time polymerase chain reaction (RT-PCR). To study the levels of gene expression of miR- 221, and VEGF, unique primers were created for each target. The online program Primer3Plus (https://www.primer3plus.com) was used to design the primers. The reference sequences came from the National Centre for Biotechnology Information (NCBI) database. The probes and primers were fine-tuned to make sure they were specific and worked well for the next RT-qPCR tests. The included variants were intronic SNPs of the MTHFR gene (rs1801133) G-for-A and (rs1801131) T-for-G. The detecting primers used for Gene expression of miR-221, and VEGF are given in Table 1. The primer efficiency and specificity were tested using the in-silico PCR online-built-in analysis (https://genome.ucsc.edu/cgi-bin/hgPcr).

Table 1: Sequences of genes expression

Primer Name	Primer Sequence F	Primer Sequence R	Tm.	Ref.
miR-221_-5p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACAAATCTA		60	1
miR-221_-3p-RT	GTGCAGGGTCCGAGGTCAGAGCCACCTGG GCAATTTTTTTTTTGAAACC			
miR-16-1_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATT CGCACCAGAGCCAACCGCCAAT			

miR-221-5p	GGTTTTTTACCTGGCATACAATGT	GTGCAGGGTCCGAGGTATT	60	
miR-221-3p	TGGAGCTACATTGTCTGCTG	AGCUACAUUGUCUGCUGGGUUUC		
miR-16	GGTTTTTTTGTAGCAGCACGTAAAT	GTGCAGGGTCCGAGGTATT	60	
VEGF	CCACACCATCACCATCGACA	TGTATGTGGGTGGGTGTGTC	60	
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG		

MTHFR primers and probes

-SNP rs1801133 forward and reverse primer were 5'- TGCCTTCACAAAGCGGAAGA-3'and 5'-GTGCTGTTGGAAGGTGCAAG-3', respectively (amplification size: 271 bp) as shown Figure1. Probes 1 and 2 were (FAM)-5' ATGAAATCGGCTCCCGCA-3'-(BHQ) and (JOE)-5' GAAATCGACTCCCGCAGACA-3'-(BHQ), respectively.

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>chr1:11796260+11796530 271bp TGCCTTCACAAAGCGGAAGA GTGCTGTTGGAAGGTGCAAG
TGCCTTCACAAAGCGGAAGAatgtgtcagcctcaaagaaaagctgcgtga
tgatgaaatcggctcccgcagacaccttctccttcaagtgcttcaggtca
gcctcaaagctccctgcttcggggtggcctttggggtaacctgccaatag
ggatgacagtcaggagaggctggcctccacctgttcaaggcgagggatga
agagaccacagggactgggcagagagagtcctctgctttgggggctctga
tCTTGCACCTTCCAACAGCAC
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Figure 1: In-Silico PCR testing of SNP rs1801133 primers.

-SNP rs1801131 forward and reverse primers were 5'- CCAGCATCACTCACTTTGTG-3' and 5'-AAGACACTTGCTTCACTGGTCA-3', respectively (amplification size: 271 bp) as shown Figure 2 . Probes 1 and 2 were (FAM)-5' GACACTTTCTTCACTGGTCAGC-3'-(BHQ) and (JOE)-5' GGAGTCAGGGGCAGAATTTA-3'-(BHQ), respectively.

Figure 2: In-Silico PCR testing of SNP rs1801131 primers.

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>chr1:11794344+11794637 294bp CCAGCATCACTCACTTTGTG GGAGTCAGGGGCAGAATTTA
CCAGCATCACTCACTTTGTGaccattccggtttggttctcccgagaggta
aagaacgaagacttcaaagacactttcttcactggtcagctcctcccccc
acatcttcagcagctcctccttgggggacttgctcttcaggtagaagagg
tagtagtccttcagctcccc aaaggcaggggaagaggaattgcccctggc
agaggggtgcccagaggtcagggcacactcctgacagagggcagtgccac
cacatgcccaggaggccattcctgTAAATTCTGCCCCTGACTCC
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Gene expression:
Process of real time PCR to study MTHFR gene expression was done by The Azure Cielo Real-Time PCR system according to the protocol of Luna® Universal Master Mix (Promega, USA). Based on the protocol, firstly, Master Mix was prepared and added to strip cap microtube and then the cDNA was added to it. The QRT-PCR amplification conditions were: 95°C, 3 min; 95°C, 15 sec, 60°C, 30 sec for 45 cycles.

cDNA Concentration (ng/µl)
cDNA Concentration 4-6ng/µl

Analysis Gene Expression
Relative quantification
Folding =2-ΔΔCT
ΔCT= CT gene - CT House Keeping gene
ΔΔCT= ΔCT Treated or Control – Average ΔCT Average Con.

RESULTS AND DISCUSSION

Allelic Quantitative Study of rs1801133 and rs1801131 SNP in MTHFR Gene

This study investigated two genetic variants (rs1801133 and rs1801131 SNPs) of the MTHFR gene to evaluate their association with susceptibility to DR. According to TaqMan allelic discrimination, rs1801133 showed three genotypes, namely GG, GA, and AA. Similarly, SNP rs1801131 showed three genotypes, namely TT, TG, and GG. The SNPs were identified using TaqMan real-time PCR. Each SNP has both homozygous and heterozygous genotypes.

Genotype Distribution and Allele Frequency of rs1801133 SNP

The results of statistical analysis of the rs1801133 SNP in the MTHFR gene showed significant differences between diabetic patients with DR and diabetic patients without DR. Table 2 of Genotype Frequencies results wild GG genotype in 46% of DR patients vs. 70% of DM patients, showing a statistically significant difference (P=0.047). The lower frequency in DR patients suggests that the GG genotype might have a protective effect against DR, or that DM patients are genetically predisposed to this genotype. The odds ratio (OR=0.37) further supports that GG carriers are less likely to develop DR. For, GA Genotype no significant difference was observed (30% in DR vs. 24% in DM, P=0.243). The OR = 1.682 but the P = 0.2896 which suggests that GA carriers might have a 68% higher risk of DR, but the result is not statistically reliable possibly due to limited sample size. So, there is a preliminary trend suggesting increased risk, but it is not statistically significant and requires further investigation to validate this finding. The OR=1.682 suggests a slight (but statistically insignificant) increase in risk for DR, but more data is needed to confirm any association, i.e., there is a preliminary trend suggesting increased risk, but it is not statistically significant and requires further investigation. Odds Ratio (OR) and Chi-square/P-value measure two different things. OR indicates the strength and direction of an association (risk or protection). While P-value reflects statistical significance (the probability that the result occurred by chance). An OR > 1 can coexist with a non-significant P-value for more than one reasons. Small sample size (low statistical power), if the sample size is small, the effect size (OR) may appear large, but the result lacks statistical significance due to insufficient data.

Thus, in the present table, the GA genotype has OR = 1.682 (~68% increased risk) but P = 0.2896 (non-significant) which can be Interpreted in that there might be a real trend, but the sample was too small to confirm it statistically. In addition, High data variability (wide confidence intervals), the data may be highly scattered (e.g., outliers), the OR may seem high, but the confidence intervals (CI) will be wide, leading to a non-significant P-value. Thus, the 95% CI for OR = [0.7, 4.0], the effect could range from negligible (0.7) to strong (4.0), making the result inconclusive. Finally, it may be no true effect (random fluctuation). Since, sometimes OR > 1 occurs by chance in a specific sample but disappears in larger/repeated studies.

Final Takeaway, no statistical contradiction exists in having OR > 1 with a non-significant P-value, but the finding remains uncertain. A true risk cannot be concluded unless P < 0.05 and CI excludes 1. Thence, GA genotype result is a "preliminary signal" needing further validation. For AA Genotype, highly significant difference (P=0.011), 24% in DR vs. only 6% in DM. The OR=4.95 indicates that individuals with the AA genotype have nearly 5 times higher risk of developing DR compared to DM patients. This strong association suggests that the AA variant may contribute to retinal vascular damage, possibly due to impaired folate metabolism and elevated homocysteine levels. Dominant model (AA vs. GG+GA) appears significant association (OR = 2.83, P = 0.011), suggesting A-allele carriers have ~2.8x higher DR risk. In

addition, recessive model (GA+AA vs. GG) appears highly significant (OR = 4.95, P = 0.011), confirming AA genotype as a ~5x higher risk genotype. Allele Frequency Analysis indicate that G allele present in 82% of DR patients vs. 61% of DM patients, but the difference was highly significant (P=0.0017). The OR=0.343 suggests that the G allele is less frequent in DR, reinforcing its potential protective role. While, A allele although not explicitly stated, the A allele appears more frequent in DR patients (39% vs. 18% in DM). Thus, A allele was strongly associated with DR (OR = 2.94, P = 0.00017). The strong association with the AA genotype suggests that the A allele may be a risk factor for DR when homozygous. Biological implications suggested that the MTHFR gene is crucial for folate metabolism, and variation (like rs1801133) can lead to elevated homocysteine, a known risk factor for microvascular complications. The AA genotype likely results in reduced enzyme,activity,increasing homocysteine levels and contributing to retinal vascular damage.

Table 2 : Genotype and allele distribution of the rs1801133 polymorphism in the MTHFR gene between diabetic patients with retinopathy (DR) and diabetic patients without (DM).

Genotypes	DR Patients) N0. (%)	DM Patients (+ve) control N0. (%)	Chi-square	P-value	O.R. (C.I)	P-value
GG	23 (46%)	35 (70%)	3.936	*0.047	0.37 (0.14-0.99)	0.0477
GA	15 (30%)	12 (24%)	1.364	0.243 NS	0.54 (0.70-4.03)	0.2896
AA	12 (24%)	3 (6%)	6.533	*0.011	4.95 (1.30-18.87)	0.0187
Dominant (GA+AA) vs.GG	27 (54%)	15 (30%)	6.4	*0.011	2.83 (1.23-6.51)	0.014
Recessive (GA+GG) vs. GA	12 (24%)	3 (6%)	6.533	*0.011	4.95 (1.30-18.87)	0.0187
Allele	**Allele frequency**					
G	0.61	0.82	9.815	*0.0017	0.343 (0.18-0.66)	0.0016

A	0.39	0.18	9.815	*0.0017	2.94 (1.52-5.68)	0.0016

* (P≤0.05), Significant difference. ** (P≤0.01), highly significant difference.

Comparison of genotypes (GG, GA, AA Genotypes) and G or A alleles between DM Patients and healthy controls in Table 3 reflect no significant differences between DM patients and healthy controls (all P > 0.05) suggesting that the rs1801133 polymorphism does not significantly associate with DM itself.
The key findings of Table 3 are that non-Significant results (P > 0.05) with OR > 1. The GA (OR=1.68) and AA (OR=1.53) genotypes show nominal risk increases for DM, but the results are not statistically significant due to small sample size, which limited power to detect true effects (especially for rare AA genotype); wide confidence intervals in GA genotype with OR=1.68 (95% CI: 0.49–3.26), range includes 1 (null effect). In addition, AA genotype appear OR=1.53 (95% CI: 0.24–9.59) which is extremely wide due to only 3 vs. 2 cases. Random variability, thus OR > 1 could occur by chance in under-powered studies. OR > 1 Despite Non- Significant P-Values can be justified as OR measures effect size, while P-value measures precision. For GA genotype, the 24% vs. 20% frequency suggests a trend, but the small sample leads to high uncertainty (P=0.629). Also, when comparing clinical vs. statistical significance, even if OR > 1, the effect may be too small or noisy to reach statistical significance. In allele-level analysis, the G-allele frequency was similar between groups (82% vs. 86%, P=0.563). A-allele showed a non-significant increase in DM (OR=1.35, P=0.563), aligning with genotype trends. From the results of table 2 and 3, the rs1801133 polymorphism does not show significant differences between DM patients and healthy controls, suggesting its role is *specific to DR progression*, not diabetes onset.

Table 3: Distribution of genotypes and alleles for the rs1801133 polymorphism in the MTHFR gene in diabetic patients (DM) vs. healthy controls.

Genotypes	DM Patients +ve control N0. (%)	(-ve) control N0. (%)	Chi-square	P-value	O.R. (C.I)	P-value
GG	35 (70%)	38 (76%)	0.457	0.499	0.737 (0.30-1.79)	0.499
GA	12 (24%)	10 (20%)	0.233	0.629	1.68 (0.49-3.26)	0.629
AA	3 (6%)	2 (4%)	0.211	0.646	1.532 (0.24-9.59)	0.646

Dominant	15 (30%)	12 (24%)	0.457	0.499	1.36 (0.54-3.41)	0.510
Recessive	3 (6%)	2 (4%)	0.211	0.646	1.53 (0.24-9.59)	1.000
Allele	**Allele frequency**					
G	0.82	0.86	0.335	0.5628	0.742 (0.35-1.59)	0.5634
A	0.18	0.14	--	--	1.35 (0.50-3.60)	--

($P < 0.05$), Non-significant difference.

The results of this study support an association between the rs1801133 polymorphism in the MTHFR gene and the risk of diabetic retinopathy. The significantly increased frequency of the AA genotype in the DR group, along with the corresponding decrease in the frequency of the GG genotype, suggest that the A mutation (representing the A allele) may directly contribute to the development of retinal vascular damage in diabetic patients.

The MTHFR gene plays an essential role in the methylation cycle, enabling the transformation of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, which is critical for the conversion of homocysteine to methionine. The mutation (AA variant) leads to a decrease in enzyme activity by roughly 60–70%, resulting in the accumulation of homocysteine in the bloodstream, a condition termed hyperhomocysteinemia (Frosst, 1995; Gui, 2020).

Hyperhomocysteinemia markedly exacerbates oxidative stress and inflammation in the microvasculature. This causes reactive oxygen species (ROS) production, reduced nitric oxide availability and permeability of the vascular. This malfunction of retinopathy causes impairment of endothelial cells, loss of capillary support and it triggers retinal apoptosis. (Kowluru, 2020; Asadi, 2024).

Consistent with these results, Abdallah et al. (2017) reported a higher frequency of the TT (AA- equivalent) genotype in Diabetic retinopathy patients when compared with controls, thereby corroborating the results obtained here. Luo et al. (2016) also displayed in a meta-analysis of several Asian studies that individuals carrying the TT pattern have strikingly elevated risk of Diabetic retinopathy. A recent meta-analysis by Zhang et al. (2024) reported that MTHFR deficiency causes activation of NF-κB and HIF-1α, pathways involved in the development of inflammation and tissue damage at the level of retina.

Conversely, several studies did not detect an association between the C677T mutation and retinopathy, as seen by Maeda et al. (2015), who attributed this discrepancy to ethnic variation and dietary folate intake. Niu et al. (2017) study and did not find an association with other genotypes, therefore it is particularly important that genetic effect is studied together with environmental factors.

In the Diabetic retinopathy and Control group, no such substantial differences were observed which affirmed that the effect of mutation rs1801133 is closely linked to development of complications as opposed

to merely presence of diabetes as mentioned by Pathak et al. (2022). Reduced MTHFR activity also results in an oxidative stress related modificative in the gene transcription involving cells are said to compensate for reduced Muller et al.,,Dermal fibroblasts (Fig. 2). (2023). This would explain higher levels of gene expression recorded higher in Diabetic retinopathy, as supported with reciprocal studies (Sikka, 2017; Jin, 2019). One of the major findings in our study is this reduplicative outcome between Diabetic retinopathy and control groups, indicating that the effect of rs1801133 mutation relates not only to the onset of diabetes but also to complications as it was reported by Pathak et al. (2022).

Moreover, diminished MTHFR activity results in alterations in gene transcription linked to oxidative stress, and it is posited that cells recompensate for this deficiency by highly regulating mRNA expression, as elucidated by Chen et al. (2023). This may elucidate the heightened gene expression levels in the DR group, as documented in analogous studies (Sikka, 2017; Jin, 2019).

This accumulating evidence supports those mutations in the MTHFR gene, by raising homocysteine levels and creating an oxidative inflammatory environment, play a pivotal role in the molecular pathogenesis of diabetic retinopathy

Genotype Distribution and Allele Frequency of rs1801131 SNP

Analysis of the rs1801131 polymorphism in the MTHFR gene for genotypes (TT, TG, GG) revealed no statistically significant differences, as all P values exceeded 0.05. This indicates a lack of direct association between these genotypes and the incidence of diabetic retinopathy. Analysis of allele frequency revealed statistically significant differences in the T allele frequency between diabetic patients with retinopathy and those without, indicated by a P value of 0.0206 and an odds ratio of 0.460 (95% CI: 0.25–0.86).

Table 4: Genotype and allele distribution of the rs1801131 polymorphism in the MTHFR gene between diabetic patients with retinopathy (DR) and diabetic patients without DR (DM).

Genotypes	DR Patients N0. (%)	DM Patients +ve control N0. (%)	Chi-square	P-value	O.R. (C.I)	P-value
TT	22 (44%)	33 (66%)	0.525	0.4689	0.733(0.34-1.58)	0.4684
TG	18 (36%)	12 (24%)	1.992	0.1583	2.045(0.83-6.02)	0.1471
GG	10 (20%)	5 (10%)	2.209	0.1373	2.667 (0.86-0.1143)	0.1143
Allele	**Allele frequency**		--	--	--	--
T	0.73	0.78	5.357	0.0206 *	0.460 (0.25-0.86)	0.0202 *
G	0.27	0.22	--	--	--	--

* (P≤0.05), Significant difference

The analysis of the rs1801131 polymorphism in the MTHFR gene showed no statistically significant differences between diabetic patients without retinopathy and healthy controls. The distribution of TT, TG, and GG genotypes was comparable between the two groups. The TT genotype represented 66.0% of DM patients and 74.0% of controls, whereas the GG genotype comprised 10.0% and 8.0%, respectively. The frequency of the T and G alleles did not show significant differences, with the T allele frequency in the DM group at 78.0% compared to 83.0% in the control group (P = 0.4753).

Table 5 : Distribution of genotypes and alleles for the rs1801131 polymorphism in the MTHFR gene between diabetic patients (DM) and healthy controls.

Genotypes	DM Patients +ve control N0. (%)	(-ve control) N0. (%)	Chi-square	P-value	O.R. (C.I)	P-value
TT	33 (66%)	37 (74%)	0.429	0.5127	0.682 (0.29-1.61)	0.5131
TG	12 (24%)	9 (18%)	0.241	0.6234	1.439 (0.55-3.80)	0.6242
GG	5 (10%)	4 (8%)	0.000	1.0000	1.278 (0.32-5.07)	1.0000
Allele	Allele frequency		--	--	--	--
T	0.78	0.83	0.510	0.4753 NS	0.726 (0.36-1.47)	0.4756 NS
G	0.22	0.17	--	--	--	--

(P > 0.05), Non-significant difference.

The results show that type 2 diabetes does not directly follow the MTHFR gene mutation rs18011331 ("T to G"). Diabetics without retinopathy and the control group had no variations in genotype or allele frequencies, according to Khan et al. (2023). Those without retinopathy and those with retinopathy showed many differences. From 78% in DM to 73% in DR, the frequency of the T (basal) allele dropped (P = 0.0206, odds ratio 0.460). This finding suggests that the T allele could stop diabetic retinopathy (Garcia-Hernandez et al., 2019) from developing. MTHFR changes homocysteine to methionine by means of 5,10-methylene- tetrahydrofolate, hence generating 5-methyl-THF (Friedman et al., 1999). In the heterozygous condition, the T>G mutation at position 1298 (A1298C) replaces glutamic acid with alanine, therefore reducing enzyme activity; in the homozygous form, this modification may be much more reduced. Diabetics without retinopathy and healthy controls found no appreciable difference in the MTHFR gene variation

rs1801131. At 8.0% and 10.0%, the TT, TG, and GG genotypes were similar throughout groups. in those with diabetes, 66.0% had TT genotypes; in the control group, 74.0% showed same. In the DM group, the frequencies of the T and G alleles were 78.0% and 83.0%, respectively (p = 0.4753). Diabetics without retinopathy and healthy controls exhibited no appreciable variations in the MTHFR gene variant rs1801131. Ten percent of diabetes mellitus patients showed the GG genotype and sixty-six percent of controls displayed the TT genotype, so the proportion of TT, TG, and GG genotypes was similar. In the DM group, the frequencies of the T and G alleles were 78.0% and 83.0%, respectively (p = 0.4753). Diabetics without retinopathy and healthy controls exhibited no appreciable variation in the MTHFR gene variant rs1801131. TT, TG, and GG genotypes had similar frequencies; 66.0% of controls showed the TT genotype, while 10.0% of diabetes mellitus patients showed the GG genotype. Whereas in the control group the p-value was 0.4753, in the DM group 78.0% showed the T allele. 2020's low homocysteine levels caused by the deficit caused oxidative stress in retinal arteries and microvessel endothelium (Kowluru and Mishra 2020). Rising homocysteine levels, according to Gu et al. (2023), could reduce nitric oxide generation, cause retinal cell death, and set off inflammatory pathways, including NF-κB.

Reduced enzyme effectiveness from the mutant G allele increases plasma homocysteine levels. The T allele is rare in DR, thereby perhaps reducing the genetic protection allowing enzyme activity (Daghlas et al., 2025).

Jain et al. (2021) confirmed our findings that compared diabetes patients with controls by reporting no connection between A1298C and diabetes risk. Rykov et al. (2022) looked at how the rs1801131 mutation affected diabetic retinopathy and found a complicated interaction between environmental and genetic elements, most importantly related to nutrition, lifestyle, and folate levels. When present alongside other variants, the rs1801131 mutation may lower MTHFR activity even if it has less influence than the more well-known rs1801134 mutation (Sienkowicz-Szłapka et al., 2023).

VEGFR expression

Table 6 indicates that the mean Ct value for the VEGF-2 gene in the disease group was 18.91, while the mean Ct values for the positive and negative control groups were 18.24 and 18.22, respectively. Upon calculating ΔΔCt, the disease group had the highest result (1.53), indicating their gene expression was 3.53 times greater than that of the negative control group. In contrast, the positive control group had reduced expression (only 0.98-fold).

The findings indicate that the VEGFR-2 gene is overexpressed in diabetic retinopathy patients compared to those without the condition, which may be linked to retinal vascular changes caused by diabetic microvascular complications. Previous studies have indicated that VEGFR-2 is a gene associated with angiogenesis, the process of development of new blood vessels, and it significantly contributes to diabetic retinopathy by enhancing vascular endothelial proliferation and permeability (Gui et al., 2020; Mohamed et al., 2020). Research, like that of Jenkins et al. (2015), has established that the overexpression of VEGFR is closely associated with the advancement of retinopathy and serves as a significant biomarker for the decline in visual function in diabetic individuals.

Table 6: Fold of VEGF expression depending on 2- □□CT method.

	Mean Ct of VEGF	Mean Ct of GAPDH	means □CT Target (CT of VEGF - Ct of GAPDH)	□CT Calibrator	□□CT (□CT - □CT Calibrator)	2-□□CT	Experiment group / control group	Fold of gene expression

Patient	18.91	23.53	-4.62	-4.01	-0.61	1.53	1.53/0.43	3.53
Control positive	18.24	23.49	-5.25	-4.01	-1.24	0.42	0.42/0.43	0.98
Control negative	18.22	23.44	-5.22	-4.01	-1.21	0.43	0.43/0.43	1.00

Results from Table 7 are indicative that the ΔC t method has been employed to assess the VEGFR-2 gene expression with reference to that of those for the GAPDH gene. The relative expression of the disease group was 1.52 times higher than those from control group, and a lower Fc value was -4.62, which meant that the genes had high level of gene expression. Diabetic patients without retinopathy showed reduced gene expression (0.98 times) in comparison with a healthy control group (1.00). The findings were consistent with the two group comparisons' results of the 2-ΔCt method, elucidating that there was a significant difference among gene expression in groups. This indicates that VEGFR-2 gene is closely related to retinal pathological changes in diabetic patient and represents an important target for the treatment of these patients at the late stage.

The population of patients manifesting diabetic retinopathy serves as an example of elevated VEGFR-2 expression, in accord with the majority of recent studies showing that up-regulation of VEGFR expression facilitates abnormal blood vessel formation and leads to increased vascular permeability. The possible consequence of this could lead to fluid and blood leakage in retina, which ultimately affects the vision (Wang et al., 2024). The VEGF and its receptor played a central role in the treatment of DR according to the American Society of Retinal Medicine diseases guideline on DR. Palaportadin, bevacizumab and ranibizumab have proven effectiveness to slow vision loss down and improve best corrected visual acuity.(Diabetic Retinopathy Clinical Research Network, 2021; Pessoa, 2022).

Table 7 : Fold of VEGFR in expression Depending on □CT (normalization Ct values) and 2-□□CT Method.

	Mean Ct of VEGFR	**Mean Ct of GAPDH**	**□CT (Means CT of VEGFR - Mean CT of GAPDH)**	**2- □CT**	**Experiment group / control group**	**Fold of gene expression**
Patient	18.91	23.53	-4.62	0.041	0.041/0.027	1.52
Control positive	18.24	23.49	-5.25	0.026	0.026/0.027	0.98
Control negative	18.22	23.44	-5.22	0.027	0.027/0.027	1

The results of the composite figure indicated that the three groups had markedly distinct levels of VEGFR-2 gene expression. In comparison to the diabetic cohort without retinal and the healthy cohort, the mean fold of gene expression was significantly elevated in patients with diabetic retinopathy, exhibiting a high degree of statistical significance. This disparity indicates that VEGFR-2-associated angiogenic pathways are differently active. This gene is recognized for its essential role in stimulating endothelial cell proliferation and enhancing vascular permeability, primarily through the activation of the VEGFR-2 receptor in retinal vascular cells. Khalaf et al. (2017) postulate that this causes pathological reorganization of the vascular network with fibrosis or bleeding during later stages of the disease. The linear expression gradient of the profile observed in normal and damaged tissues supports that this gene is strongly up-regulated by localized stimuli only in the retinal tissue, for instance triggered by local inflammation or hypoxia in retina tissue. This is consistent with the conjecture of Xia et al. (2022) who showed that VEGFR-2 is stimulated by prolonged ischemia to induce activation of HIF-1α in retinal cells through different signaling regulations. All four sample groups had a range of individual expression levels; the retinopathy patients group, however, exhibited a higher median distribution, which represented a more uniform gene response within this group. These results suggested that changes in expression may occur during the development of minor complications related to diabetes on the retinal vessels. Data such as this (Homme et al., 2018) reveal a link between increased expression of VEGF-2 and the extent of retinal capillaropathy by the retina, so there is support for that conclusion.

The graph distribution also showed that change in gene expression was not random, but rather indicated a state of pathophisology associated with the disease severity. This seems to indicate the possibility of VEGF-2 as a biomarker for early diagnosis and evaluation of the diabetic retinopathy at various stages. Colamonici et al. (2021) demonstrated that also in the quantitative distribution of values in the disease group, not only a response occurs but there is likely genetic predisposition toward a reflection of an aggregation influence on inflammatory and chronic hypoxic patterns. New findings about VEGFR-2 inhibitors, which are being used in some mode of treatment for diabetic retinopathy, including the report by Wang et al. (2024) in terms of targeted therapies such as Aflibercept and Bevacizumab, collectively propose targeting VEGFR-2 itself or its regulatory pathways may be beneficial therapeutically.

miRNA-221expression

Three groups were utilized to evaluate miRNA-16 gene expression: the patient group, comprising diabetics with retinopathy; the positive control group, consisting of diabetics without retinopathy; and the negative control group, composed of healthy individuals. The Ct value of the housekeeping gene miRNA- 16 used in the present study is shown in table 8. The expression levels of the miRNA-16 gene in these groups are compared. The results indicated that, with an expression value of 0.94, the patients' miRNA-16 gene expression levels were slightly lower than those of the negative control group. Relative to the negative control, designated as the reference standard (1.00), the positive control group had a negligible increase (1.04 times).

Table 8: Comparison of miRNA-16 Fold expression between study exposure groups

	Means Ct of miRNA-16	2-Ct	Experiment group / control group	Fold of gene expression
Patient	25.11	2.761E-08	2.761E-08/2.939E-08	0.94

Control positive	24.96	3.064E-08	3.064E-08/2.939E-08	1.04
Control negative	25.02	2.939E-08	2.939E-08/2.939E-08	1.00

A microregulatory gene, miRNA-16 has been demonstrated to be rather stable across several cell types and tissues. Its steady basal activity inside the cell shows it, usually remaining unaffected by pathogenic or environmental influences, hence it is often used as an internal reference gene (housekeeping gene) in RT-qPCR gene expression studies (Peltier and Latham, 2008).
By confirming its validity as a consistent internal control gene for calibrating the expression of other genes, including miRNA-221, this study revealed no notable difference in the Ct value of miRNA-16 among the three groups—patients, positive control, and negative control. Many tissues and disease states have been shown in several studies to exhibit steady miRNA-16 expression, making it suitable for gene expression level comparison free of analytical bias (Schwarzenbach et al., 2015).
Rebouças et al. (2013) emphasized that reference genes included in gene expression studies must show relative stability across all physiologic and pathological conditions investigated, a criterion fulfilled by miRNA-16 based on various comparative evaluations.
The expression of miRNA-221 was evaluated using the 2-ΔΔCt method across three study cohorts: the patient group (diabetic persons with retinopathy), the positive control group (diabetic individuals without retinopathy), and the negative control group (healthy subjects). Table 9 presents the gene expression levels of miRNA-221 across these categories.

The findings indicated that the expression level of miRNA-221 in the patient cohort was significantly elevated (5.94-fold) relative to the negative control group, which serves as the baseline (1.00-fold), whereas the expression in the positive control group was marginally increased (1.61-fold) compared to the negative control.

Table 9 : Fold of miRNA-221 expression in Depending on 2-□□CT method

	Mean Ct of MTHFR	**Mean Ct of GAPDH**	**means □CT Target (CT of MTHFR - Ct of miRNA-16)**	**□CT Calibrator**	**□□CT (□CT - □CT Calibrator)**	**2-□□CT**	**Experiment group/ control group**	**Fold of gene expression**
Patient	28.06	25.11	2.95	3.04	-0.09	0.94	0.94/0.16	5.94
Control positive	29.97	24.96	5.01	3.04	1.97	0.26	0.26/0.16	1.61

Control negative	30.72	25.02	5.7	3.04	2.66	0.16	0.16/0.16	1.00

miRNA-221 significantly regulates various physiological functions, including inflammation, cell proliferation, and oxidative stress response (Di Leva et al., 2014). The markedly increased expression levels of miRNA-221 in diabetic patients with retinopathy indicate its possible involvement in the pathogenic mechanisms linked to diabetic retinopathy.

Prior research suggests that miRNA-221 might augment the expression of specific inflammatory mediators, including VEGFR, a critical element in the progression of vascular and retinal injury in diabetic individuals (Polu and Lowman, 2014). The findings align with the current study's results, corroborating the idea that miRNA-221 is linked to the advancement of diabetic retinopathy. The modest increase in the positive control group (diabetics without retinopathy) indicates that alterations in gene expression of this molecule are specifically associated with retinopathy rather than diabetes alone, aligning with findings from other studies (Shantikumar et al., 2012). The findings contradict several other studies that have indicated reduced levels of miRNA-221 at certain stages of diabetes, and this inconsistency may stem from the varying phases of the disease examined (Poliseno et al., 2010).

The ΔCt technique was employed to assess miRNA-221 gene expression across three cohorts: the patient group (diabetics with retinopathy), the positive control group (diabetics without retinopathy), and the negative control group (healthy persons).

Table 10 presents the gene expression levels across the examined groups. The results indicate that miRNA-221 gene expression in the patient group was 6.73 times more than in the negative control group, designated as the standard (1.00 times), whilst the positive control group exhibited a modest rise of 1.61 times.

Table 10: Fold of miRNA-221 in expression Depending on Δ CT (normalization Ct values) and 2-ΔΔ CT Method.

	Mean Ct of miRNA -221	**Mean Ct of miRNA -16**	**□CT (Means CT of miRNA-221 - Mean CT of miRNA-16)**	**2- □CT**	**Experiment group / control group**	**Fold of gene expression**
Patient	28.06	25.11	2.95	0.13	0.13/0.02	6.73
Control positive	29.97	24.96	5.01	0.03	0.03/0.02	1.61
Control negative	30.72	25.02	5.7	0.02	0.02/0.02	1.00

The significant increase in miRNA-221 gene expression in diabetic patients with retinopathy indicates a possible involvement of this molecule in the inflammatory and oxidative processes linked to retinopathy. The results corroborate earlier research indicating that miRNA-221 facilitates the expression of critical mediators like VEGFR, potentially exacerbating disease development and causing damage to retinal

microvessels (Polu and Lowman, 2014). The modest elevation in the positive control group may signify early molecular alterations before retinopathy onset, positioning miRNA-221 as a prospective biomarker for early detection. This conclusion aligns with other studies (Shantikumar et al., 2012) but contrasts with other research that indicated reduced expression levels, maybe attributable to variations in experimental circumstances and sample types (Poliseno et al., 2010).

Conclusions:

This study shows many scientific aspects:

1-Genetic studies confirmed molecular mechanisms of diabetic retinopathy (DR)and found a strong correlation AA and GA genotype and A allele of the rs1801133 polymorphism in the MTHFR gene are significant risk factors for DR, while the GG genotype and G allele may be protective. This SNP does not influence diabetes susceptibility but is specific to DR progression. The small sample size and wide confidence intervals preclude definitive conclusions.

2- VEFG, and miR-211 gene expression changed significantly in affected individuals. As shown here, molecular analyses must be combined with conventional screening methods to better predict disease progression and identify risk factors. At-risk populations should be screened for retinal diseases using genetic and physiological markers, according to the study. This would enable early retinal damage prevention.

3-The present study is helpful to provide information to other researchers about the gene variation analyzed in Iraqi DR patients which may be helpful for tailoring therapy in clinical practice.

References:

- Cai, Y., Liu, B., Zhang, Y., and Zhou, Y. (2024). MTHFR gene polymorphisms in diabetes mellitus. Clinica Chimica Acta, 119825.
- Cao, W., Li, N., Zhang, R., Li, W., Gao, M., Wang, H., and Yu, Z. (2024). Interactive effects of gestational diabetes and high pre-pregnancy body mass index on adverse growth patterns of offspring. Diabetes/Metabolism Research and Reviews, 40(3), e3759.
- Chen, S., Saeed, A. F., Liu, Q., Jiang, Q., Xu, H., Xiao, G. G., and Duo, Y. (2023). Macrophages in immunoregulation and therapeutics. Signal transduction and targeted therapy, 8(1), 207.
- Colamonici, M. A., Epshtein, Y., Chen, W., & Jacobson, J. R. (2021). Haloperidol attenuates lung endothelial cell permeability in vitro and in vivo. Cells, 10(9), 2186.
- Di Leva, G., Garofalo, M., & Croce, C. M. (2014). MicroRNAs in Friedman, G., Goldschmidt, N., Friedlander, Y., Ben-Yehuda, A., Selhub, J., Babaey, S., ... & Bar-On, H. (1999). A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. The Journal of nutrition, 129(9), 1656-1661.
- Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews,R. G., ... & Rozen, R. (1995). A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nature genetics, 10(1), 111-113.
- Gui, F., You, Z., Fu, S., Wu, H., and Zhang, Y. (2020). Endothelial dysfunction in diabetic retinopathy. Frontiers in Endocrinology, 11, 591.
- H. S., ... & Tyagi, S. C. (2018). Remodeling of retinal architecture in diabetic retinopathy: disruption of ocular physiology and visual functions by inflammatory gene products and pyroptosis. Frontiers in Physiology, 9, 1268.
- Huo, Y., Zhang, X., Su, L., and Zhang, Y. (2024). Association of VEGF+ 936 C/T Polymorphism with Susceptibility to Type 2 Diabetic Retinopathy: A Meta-Analysis. Hormone and Metabolic Research.
- Jain, D., Joshi, R., Dhuria, D. and Sain, M. (2021). Evaluation of efficacy of autoclave and microwave for sterilization of biomedical waste. The Pharma Innovation Journal 18(7), pp. 18–20.
- Jiang, H., Liu, Z., Townsend, J. H., and Wang, J. (2023). Effects of Methylenetetrahydrofolate Reductase (MTHFR) Polymorphisms on Retinal Tissue Perfusion in Mild Diabetic Retinopathy Patients

Receiving the Medical Food, Ocufolin®. Clinical Ophthalmology, 1121-1127.

- Jin, J., Wang, X., Zhi, X., & Meng, D. (2019). Epigenetic regulation in diabetic vascular complications. Journal of Molecular Endocrinology, 63(4), R103-R115.
- Khalaf, N., Helmy, H., Fahmy, I., Abd El Hamid, M., & Moemen, L. (2017). Role of angiopoietins and Tie-2 in diabetic retinopathy. Electronic physician, 9(8), 5031.
- Khan, N., Paterson, A. D., Roshandel, D., Maqbool, S., Fazal, N., Ali, L., … & Qamar, R. (2023). Role of 19 SNPs in 10 genes with type 2 diabetes in the Pakistani population. Gene, 848, 146899.
- Kowluru, R. A. (2020). Retinopathy in a diet-induced type 2 diabetic rat model and role of epigenetic modifications. Diabetes, 69(4), 689-698.
- Kowluru, R.A.,Mohammad,G., &Sahajpal,N.(2020).Faulty homocysteine recycling in diabetic retinopathy. Eye and vision, 7, 1-11.
- Mishra, B., Swaroop, A., and Kandpal, R. P. (2020). Genetic components in diabetic retinopathy. Indian journal of ophthalmology, 64(1), 55-61.
- Moin, M., Saha, A., Bakshi, A., Madhav, M., and Kirti, P. (2021). Constitutive expression of ribosomal protein L6 modulates salt tolerance in rice transgenic plants. Gene, 789, 145670.

- Pathak, D., Shrivastav, D., Verma, A. K., Alsayegh, A. A., Yadav, P., Khan, N. H., ... & Beg, M. M. A. (2022). Role of metabolizing MTHFR gene polymorphism (rs1801133) and its mRNA expression among Type 2 Diabetes. Journal of Diabetes & Metabolic Disorders, 21(1), 511-516.
- Peltier, H. J., & Latham, G. J. (2008). Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. Rna, 14(5), 844-852.
- Pessoa, B. B. D. S. T. (2022). The Role of Vitreous and Vitreoretinal Interface in the Management of Diabetic Macular Edema (Doctoral dissertation, Universidade do Porto (Portugal)).
- Poliseno, L., Tuccoli, A., Mariani, L., Evangelista, M., Citti, L., Woods, K., ... & Rainaldi, G. (2010). MicroRNAs modulate the angiogenic properties of HUVECs. Blood, 108(9), 3068-3071.
- Polu, K. R., & Lowman, H. B. (2014). Probody therapeutics for targeting antibodies to diseased tissue. Expert opinion on biological therapy, 14(8), 1049-1053.
- Rebouças, E. D. L., Costa, J. J. D. N., Passos, M. J., Passos, J. R. D. S., Hurk, R. V. D., & Silva, J. R. V. (2013). Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. Brazilian Archives of Biology and Technology, 56, 143-154.
- Rykov, S. O., Prokopenko, I. V., Natrus, L. V., & Panchenko, I. O. (2022). Role of polymorphisms of folate-cycle enzymes in diabetic retinopathy Ophthalmology (Ukraine)/Oftal′mologičeskij Žurnal, (5).
- Schwarzenbach, H., Da Silva, A. M., Calin, G., & Pantel, K. (2015). Data normalization strategies for microRNA quantification. Clinical chemistry, 61(11), 1333-1342.
- Shantikumar, S., Caporali, A., & Emanueli, C. (2012). Role of microRNAs in diabetes and its cardiovascular complications. Cardiovascular research, 93(4), 583-593.
- Sienkiewicz-Szłapka, E., Fiedorowicz, E., Król-Grzymała, A., Kordulewska, N., Rozmus, D., Cieślińska, A., and Grzybowski, A. (2023). The Role of Genetic Polymorphisms in Diabetic Retinopathy: Narrative Review. International Journal of Molecular Sciences, 24(21), 15865.
- Smoleński, M., Karolewicz, B., Gołkowska, A. M., Nartowski, K. P., and Małolepsza-Jarmołowska, K. (2021). Emulsion-based multicompartment vaginal drug carriers: from nanoemulsions to nanoemulgels. International Journal of Molecular Sciences, 22(12), 6455.
- Wang, M., Li, Q., Jin, M., Wang, Z., Zhang, X., Sun, X., and Luo, Y. (2022). Noncoding RNAs Are Promising Therapeutic Targets for Diabetic Retinopathy: An Updated Review (2017–2022). Biomolecules, 12(12), 1774.
- Wang, Y., Sun, X., Xie, Y., Du, A., Chen, M., Lai, S., ... and Wang, C. (2024). Panax notoginseng saponins alleviate diabetic retinopathy by pathway. Journal of Ethnopharmacology, 319, 117.