Original Article

MicroRNA Profiles Targeting Angiopoietin-1, Angiopoietin-2, and TEK Receptor Tyrosine Kinase-2 Genes Associated with Angiogenesis in Proliferative Diabetic Retinopathy

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Abstract

Objectives: This study aims to assess the concentrations of angiopoietin-1, angiopoietin-2, and Tie-2, which are implicated in the pathophysiology of proliferative diabetic retinopathy (PDR), in the vitreous fluid and to evaluate the expression profiles of microRNA (miR)-145-5p, miR-542-3p, miR-5195-3p, miR-126-3p, miR-211-3p, and miR-204-5p.

Materials and Methods: The study included 25 patients with PDR and 25 non-diabetic individuals as controls. Vitreous angiopoietin-1, angiopoietin-2, and Tie-2 levels were measured using enzyme-linked immunosorbent assay (ELISA). miRNA expression levels were evaluated using real-time polymerase chain reaction.

Results: Vitreous angiopoietin-1 and 2 levels were significantly lower in the PDR group when compared to controls ($p<0.05$). The PDR group also had a lower angiopoietin-1/angiopoietin-2 ratio and higher Tie-2 levels, but these differences did not reach statistical significance ($p>0.05$). Significantly higher levels of miR-126-3p and miR-204-5p were detected in the PDR group ($p<0.05$), whereas miR-211-3p, miR-5195-3p, miR-542-3p, and miR-145-5p did not show statistically significant differences ($p>0.05$).

Conclusion: Our data demonstrate that increased miR-204-5p and miR-126-3p expression may be associated with angiogenesis-related alterations in PDR. These findings provide insight into PDR-related angiogenesis and suggest that these microRNAs may represent potential biomarkers of disease-related vascular alterations.

Keywords: Proliferative diabetic retinopathy, microRNA, angiopoietin-1, angiopoietin-2, receptor tyrosine kinase-2

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Introduction

As a microvascular complication of diabetes, diabetic retinopathy (DR) results in substantial visual consequences in adults.[1] Clinically, DR progresses from a non-proliferative stage to a proliferative form. Non-proliferative diabetic retinopathy (NPDR) involves microvascular alterations confined to the retina, whereas proliferative diabetic retinopathy (PDR) reflects an advanced disease stage involving aberrant intraocular vessel growth and the development of fibrovascular tissue at the vitreoretinal interface. Both stages contribute substantially to irreversible vision loss.[2]

The pathogenesis of PDR is closely linked to retinal ischemia and the subsequent upregulation of angiogenic signaling pathways. Beyond vascular endothelial growth factor (VEGF), the angiopoietin/Tie2 (Ang/Tie2) axis is an important mediator regulating vascular stability and pathological angiogenesis in the diabetic retina.[3,4] Angiopoietin-1 (Ang-1), primarily produced by perivascular cells, activates the Tie2 receptor and promotes vessel maturation and stabilization by enhancing endothelial cell survival, intercellular adhesion, and barrier integrity.[5]

In contrast, angiopoietin-2 (Ang-2) is generally considered a context-dependent antagonist of Tie2 and contributes to vascular destabilization and pericyte dropout, thereby sensitizing the vasculature to pro-angiogenic cues.[6] Diabetic retinal stressors, particularly hypoxia, hyperglycemia, and oxidative stress, have been shown to increase Ang-2 expression, and hypoxic conditions also enhance VEGF signaling. The combined action of Ang-2 and VEGF facilitates endothelial activation, sprouting, and neovascular growth, promoting the progression of PDR.[7]

The expression of various genes is post-transcriptionally regulated by microRNAs (miRNAs), a family of non-coding RNAs.[8] These regulators recognize and bind to the 3′ untranslated regions of target transcripts, thereby hindering their translation into proteins.[9] Through these regulatory actions, miRNAs participate in a wide range of biological mechanisms, including angiogenic regulation; cellular differentiation, proliferation, and growth; apoptosis; and embryonic development. Therefore, miRNA downregulation or dysfunction, as well as the dysregulation of miRNA targets, is believed to contribute to various diseases. Moreover, studies are ongoing to investigate the transcriptional regulation of miRNAs and their roles in several eye diseases and retinal neovascularization.[10] The abnormal expression and altered activity of numerous retinal miRNAs have been associated with the etiology of common retinal disorders, including PDR.

The involvement of miRNAs in PDR pathogenesis has attracted growing attention due to their potential utility in disease treatment and biomarker identification.[8,11] Recent meta-analyses indicate that circulating miRNAs can distinguish PDR from NPDR, although existing evidence is largely based on serum or plasma samples and does not fully capture the angiogenic microenvironment at the vitreoretinal interface.[12]

The selected miRNAs (miR-145-5p, miR-542-3p, miR-5195-3p, miR-126-3p, miR-211-3p, and miR-204-5p) were chosen based on *in silico* target prediction tools (e.g., TargetScan and miRWalk) and previous literature indicating their potential interactions with *Ang-1, Ang-2*, and *Tie-2*–related pathways, as well as their reported relevance to angiogenesis and retinal vascular disorders.

This study focuses on quantifying the vitreous expression levels of these miRNAs that target *Ang-1, Ang-2,* and *Tie2* genes, seeking to uncover the underlying molecular mechanisms of PDR in comparison to non-diabetic controls.

Materials and Methods

All experimental procedures were approved by the Trakya University Faculty of Medicine Scientific Research Ethics Committee (protocol code: TÜTF-BAEK 2020/273, decision no: 14/22, date: 14.09.2020). We enrolled 25 patients with PDR and 25 individuals without diabetes from the Department of Ophthalmology, Trakya University Faculty of Medicine. For the PDR group, HbA1c levels were measured to document glycemic status and to confirm diabetes mellitus. The diagnosis of PDR was established based on the presence of retinal neovascularization, vitreous hemorrhage, and fibrovascular proliferative tissue.

General exclusion criteria for both groups included age-related macular degeneration, a history of complicated cataract surgery, and uveitis. Additionally, individuals with diabetes mellitus or other retinal vascular disorders were excluded from the control group. For the PDR group, further exclusion criteria comprised a history of surgery for retinal detachment, epiretinal membrane, or macular hole, and having received intravitreal injections within the previous three months.

In both groups, vitreous specimens were obtained intraoperatively during pars plana vitrectomy (PPV). Because vitreous sampling from completely healthy individuals is not ethically feasible, control specimens were obtained from patients undergoing PPV for conditions not associated with retinal vascular pathology (rhegmatogenous retinal detachment, macular hole, epiretinal membrane). This approach is consistent with previously published vitreous studies, in which control samples were typically obtained from patients undergoing vitrectomy for non-vascular retinal conditions, due to ethical limitations in obtaining vitreous fluid from healthy individuals.

Vitreous Sample Collection

Each participant underwent conventional three-port 23-gauge PPV performed by the same senior surgeon under local anesthesia. At the beginning of the procedure, before initiating intraocular infusion, approximately 0.5 mL of vitreous humor was aspirated from the central vitreous cavity using a syringe attached to the vitrectomy cutter. Any residual irrigation fluid within the cutter tubing was expelled before sample collection. Vitreous samples were clarified by centrifugation and stored at -80 °C until further analysis.

Isolation of miRNA and cDNA Synthesis

After thawing the vitreous samples, total RNA including miRNAs was isolated using a commercial miRNA isolation kit (High Pure miRNA Isolation Kit, Roche Diagnostic GmbH, Mannheim, Germany). RNA quantity and purity were measured spectrophotometrically. Complementary DNA (cDNA) was synthesized from the isolated RNA using an miRNA-specific cDNA synthesis kit (miRNA All-In-One cDNA Synthesis Kit, Abm, Canada). Synthesized

cDNA was kept at -20 °C for subsequent polymerase chain reaction (PCR) analysis.

Quantitative Real-Time PCR Analysis (qRT-PCR)

The expression levels of miRNAs were analyzed using an qRT-PCR (StepOne, Applied Biosystems, USA). Normalization of miRNA expression levels was performed using RNU6B as an endogenous control. The primer sequences for the miRNAs and RNU6B are provided in Table 1. Quantitative PCR assays for miRNA expression were conducted in 20-µL reaction mixtures containing Bright Green miRNA qPCR Master Mix-No Dye (Abm, Canada), gene-specific primers, cDNA template, and nuclease-free water. qPCR amplification was conducted using standard thermal cycling parameters recommended by the manufacturer to ensure reliable and reproducible amplification.

Determination of Ang-1, Ang-2, and Tie2 Levels in Vitreous Samples

After samples were equilibrated to room temperature, enzyme-linked immunosorbent assay (ELISA)-based quantification was performed to determine vitreous levels of angiogenic pathway components, including angiopoietins and the Tie2 receptor (ANG-1 ELISA kit, Cat. no. E1222Hu BTLAB; ANG-2 ELISA kit, Cat. no. E1221Hu BTLAB; TEK ELISA kit, Cat. no. SEA126Hu, USCN, respectively). Optical density measurements were obtained with a microplate reader (Thermo Multiscan Go Microplate, USA), and concentrations of all assessed angiogenic components were expressed in ng/mL.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 25.0, IBM Corp., Armonk, NY, USA). Power calculations were conducted with PASS software targeting 80% power at α=0.05. Data normality was assessed using the Shapiro-Wilk test. Depending on distribution characteristics, comparisons were performed using the independent samples t-test or the Mann-Whitney U test. Data were presented as mean ± standard deviation or median (interquartile range), as appropriate. miRNA expression levels were normalized to RNU6B and quantified using the $2^{-\Delta\Delta Ct}$ method.[13] Correlation analyses between miRNA expression levels and angiopoietin concentrations were performed using Spearman's rank correlation coefficient. A p value <0.05 was considered statistically significant. Given that multiple comparisons were performed, the possibility of type I error cannot be excluded. No formal correction was applied due to the exploratory nature of the study and limited sample size; therefore, the findings should be considered hypothesis-generating.

Results

In the PDR group, 12 patients (48%) had vitreous hemorrhage and 13 patients (52%) had tractional retinal detachment. The mean diabetes duration in the PDR patients was 20 years (20.0±9.5). In the control group, 18 patients (72%) had rhegmatogenous retinal detachment, 4 (16%) had epiretinal membrane, and 3 (12%) had idiopathic macular hole. The study groups did not differ significantly in terms of age or gender (p>0.05). Patients with PDR exhibited higher HbA1c levels compared to controls (p<0.05). Demographic characteristics of the study population are summarized in Table 2.

Vitreous concentrations of Ang/Tie2 axis components

A significant reduction in the vitreous levels of both angiopoietins was observed in the PDR group (p<0.05; Figure 1). Assessment of the relative Ang-1 to Ang-2 ratio in vitreous samples revealed a lower value in patients with PDR (2.08±0.43 ng/mL vs. 2.20±0.58 ng/mL), although the difference was not statistically significant (p=0.412). Tie2 levels were higher in the PDR group (8.87±2.03 ng/mL) when compared with controls (8.16±2.20 ng/mL), but this increase also remained non-significant (p>0.05).

Table 1. miRNA primer sequences

Primer	Base sequence
hsa-miR-145-5p	5'-GUCCAGUUUUCCCAGGAAUCCCU-3'
hsa-miR-542-3p	5'-UCGGGGAUCAUCAUGUCACGAGA-3'
hsa-miR-5195-3p	5'-AUCCAGUUCUCUGAGGGGGCU-3'
hsa-miR-126-3p	5'-UCGUACCGUGAGUAAUAAUGCG-3'
hsa-miR-211-3p	5'-GCAGGGACAGCAAAGGGGUGC-3'
hsa-miR-204-5p	5'-UUCCCUUUGUCAUCCUAUGCCU-3'
RNU6B	5'-AACGCTTCACGAATTTGCGT-3'

Vitreous miRNA Expression Patterns in PDR and Controls

We quantified six miRNAs in PDR patients and non-diabetic controls using qRT-PCR. We found that vitreous miR-204-5p and miR-126-3p were notably increased in PDR patients relative to controls, demonstrating fold changes of 3.72 (p=0.02) and 2.63 (p=0.022), respectively. Vitreous miR-542-3p levels were approximately 1.57-fold higher in the PDR group, but this increase did not reach statistical significance (p=0.17; Figure 2). In contrast, miR-211-3p, miR-145-5p, and miR-5195-3p exhibited lower expression levels in the PDR group, again with no significant intergroup differences being identified (p>0.05; Figure 2). Spearman correlation analysis performed within the PDR group revealed no significant correlation between miR-126-3p/miR-204-5p expression levels and Ang-1/Ang-2 concentrations (p>0.05). However, a moderate positive correlation was observed between miR-126-3p and miR-204-5p expression levels (r=0.508, p=0.009). No significant correlations were observed between miRNA expression levels and Hemoglobin A1c (HbA1c) values (p>0.05)

Discussion

The present findings highlight the potential involvement of the Ang/Tie2 signaling axis in the development of PDR and suggest that selected miRNAs may be associated with alterations in the intraocular angiogenic environment rather than directly regulating these pathways. Additionally, we examined the expression profiles of miR-145-5p, miR-542-3p, and miR-5195-3p, which target the *Ang-2* gene in vitreous fluid.

In one study, the vitreous expression levels of both angiopoietins were reported to be increased in PDR patients relative to controls.[14] Similarly, Tsai et al.[15] identified elevated vitreous Ang-1 and Ang-2 concentrations in PDR patients compared to control subjects and NPDR cases. Conversely, Patel et al.[16] observed decreased levels

Table 2. Demographic parameters of control and proliferative diabetic retinopathy (PDR) groups

	PDR (n=25)	Control (n=25)	p
Age (years), mean ± SD	56.7±8.83	58.64±12.15	0.72
Sex, n (%) Male Female	 19 (76) 6 (24)	 18 (72) 7 (28)	0.76
HbA1c (%), mean ± SD	9.56±1.40	5.40±0.58	0.001

HbA1c: Hemoglobin A1c, n: Number of people, SD: Standard deviation

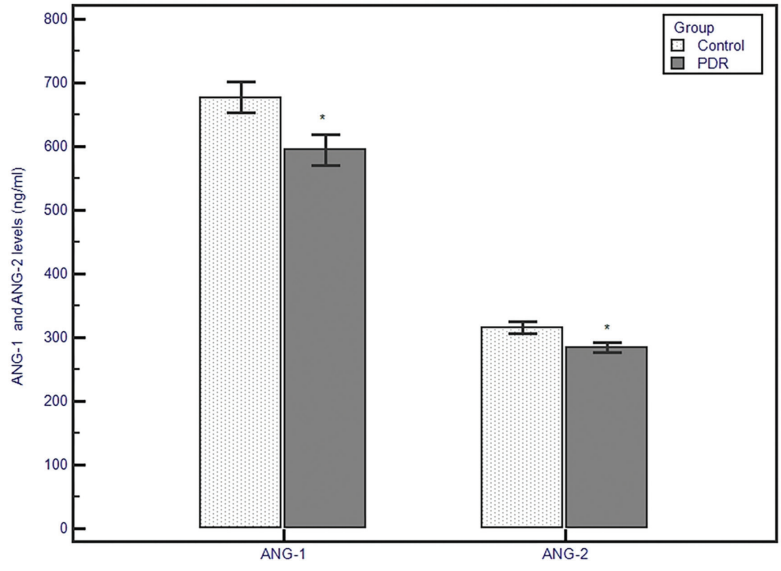

Figure 1. Vitreous concentrations of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) in patients with proliferative diabetic retinopathy (PDR) and controls
**p<0.05 compared to the control group; data are presented as mean ± SD*
SD: Standard deviation

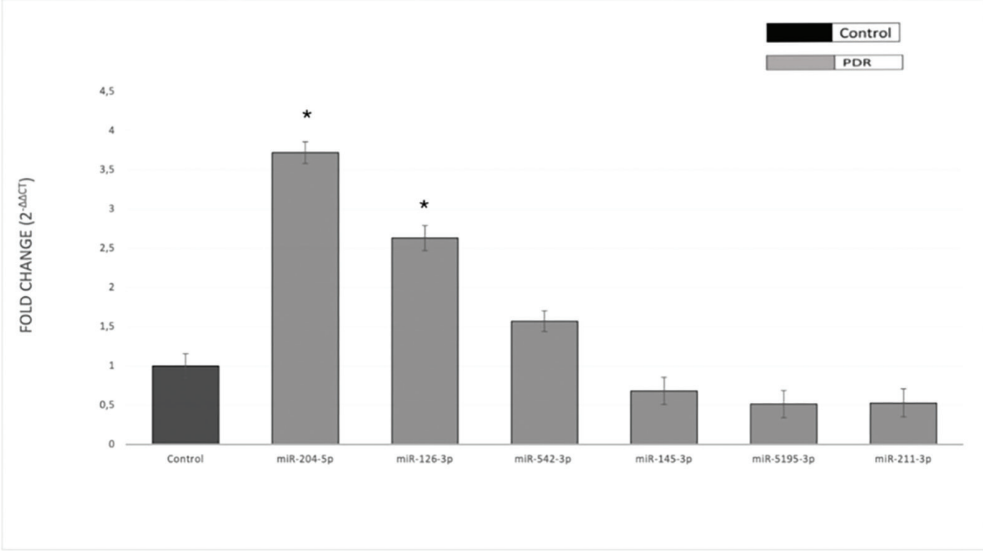

Figure 2. Relative expression levels of vitreous miRNAs in patients with proliferative diabetic retinopathy (PDR) compared to controls
**p<0.05 compared to the control group; data are presented as mean ± SD*
SD: Standard deviation

of these angiogenic factors in the vitreous fluid of PDR patients compared to the control group. The latter study is consistent with our findings of lower vitreous expression levels of both Ang-1 and Ang-2 in PDR patients relative to controls. Differences in control group composition, particularly the inclusion of eyes with non-vascular retinal disorders, may also contribute to variability in reported vitreous angiopoietin levels across studies.

Thus, the literature data regarding Ang-1 and Ang-2 regulation in the vitreous pathophysiological mechanisms of PDR are contradictory. Some studies indicate that measuring only one angiopoietin level is not sufficient to evaluate the balance in the angiogenesis process.[4,5,14] Therefore, Wang et al.[17] determined the Ang-1/Ang-2 ratio and found it was significantly decreased in PDR compared to moderately severe NPDR and more severe DR. Other studies have similarly demonstrated shift toward Ang-2 in PDR relative to controls.[4,14] A reduced Ang-1/Ang-2 ratio was also observed in the current study, consistent with the literature. Our findings support that changes in angiopoietin levels shift the balance towards neovascularization and affect the pathogenesis of PDR. The finding of reduced vitreous Ang-1 and Ang-2 levels in the PDR group contrasts with several previous studies reporting elevated levels in PDR. This discrepancy may be related to differences in disease stage, clinical phenotype, the presence of vitreous hemorrhage or tractional retinal detachment, and the heterogeneous nature of the control group. In our cohort, 48% of patients had vitreous hemorrhage and 52% had tractional retinal detachment, both of which may influence the intraocular molecular environment. Therefore, our angiopoietin findings should be interpreted within the specific clinical and methodological context of this study, and may reflect disease heterogeneity or sampling-related differences rather than a uniform biological response.

In line with this, a lower Ang-1/Ang-2 balance in patients with PDR has been associated with impaired endothelial vascular integrity. Accordingly, maintaining this balance may be important for vascular homeostasis, although its therapeutic implications require further investigation.

According to Pramanik et al.,[18] miR-126-3p levels were lower in vitreous and plasma samples from patients with NPDR than in non-DR controls. Similarly, experimental studies using streptozotocin (STZ)-induced diabetic rat models demonstrated reduced retinal miR-126-3p levels, suggesting that this miRNA may be involved in the early pathogenesis of DR by regulating the expression of VCAM-1, VEGF, and Ang-1.[19] In contrast, our study revealed a notably elevated miR-126-3p expression in the vitreous fluid of patients with PDR, accompanied by a concomitant decrease in Ang-1 levels relative to controls. Considering the protective role of Ang-1 in Tie2-mediated vascular stabilization, the observed inverse relationship between elevated miR-126-3p expression and reduced Ang-1

levels suggests a mechanism that may contribute to the maintenance of pathological angiogenesis in PDR. Our findings align with the observations of Liu et al.,[20] who reported significantly increased miR-126 expression in the vitreous, plasma, and proliferative membranes of patients with PDR, with expression levels increasing in parallel with disease severity. Other vitreous-based studies have also demonstrated upregulation of miR-126-3p in PDR compared with controls and suggested an association with increased VEGF levels and angiogenic activity.[21] Although Ang-1 levels were reduced in the same group, no significant correlation was detected between miR-126-3p expression and Ang-1 concentrations. Therefore, any potential relationship between miR-126-3p and Ang-1/Tie2 signaling in PDR remains indirect and cannot be interpreted as a causal interaction. These findings suggest that miR-126-3p may be associated with angiogenic alterations in advanced disease stages rather than directly regulating Ang-1 expression.

Regarding miR-204-5p, Kot and Kaczmarek[22] and Yan et al.[23] reported significant decreases in exosomal miR-204-5p in the vitreous of patients with PDR. In contrast, our analysis of total vitreous miRNA revealed that miR-204-5p expression was notably increased in PDR patients compared to controls. This elevation is consistent with experimental observations in STZ-induced diabetic models, where retinal miR-204-5p levels surpassed those found in non-diabetic controls, suggesting early involvement in the disease process.[24] The discrepancy between exosomal and total vitreous levels suggests that total vitreous miRNA levels may more accurately reflect retinal tissue-level pathology. Furthermore, Kather et al.[25] reported that decreased miR-204-5p expression leads to increased Ang-1 expression, thereby promoting corneal neovascularization, while Zhang et al.[26] demonstrated that Ang-1 is a direct target of both miR-204 and miR-211 in EA.hy926 endothelial cells. In the current study, miR-204-5p expression was increased in vitreous samples from PDR patients, whereas miR-211 expression showed a non-significant decreasing trend. Although experimental studies have identified Ang-1 as a potential target of miR-204-5p, we did not observe a significant correlation between miR-204-5p expression and Ang-1 levels. Therefore, the observed upregulation of miR-204-5p in PDR should be interpreted as an association rather than evidence of direct regulatory activity. Similarly, although several miRNAs evaluated in this study have been reported to target angiogenic pathways, no significant correlations were observed between miRNA expression levels and Ang-1 or Ang-2 concentrations in our dataset. This lack of association suggests that the relationship between miRNAs and angiopoietin signaling in PDR may be complex, indirect, or context-dependent, potentially involving multiple regulatory layers.

In addition, no significant correlation was identified between miRNA expression levels and HbA1c values, indicating that vitreous miRNA expression may be more strongly influenced by local intraocular mechanisms rather than systemic glycemic status.

In the present study, vitreous miR-542-3p levels showed a 1.57-fold increase in patients with PDR compared with controls, although this difference was not statistically significant ($p>0.05$). Zhang et al.[27] investigated the circSIRT2/miR-542-3p/VASH1 regulatory network in subretinal fibrosis models and elucidated the functional roles of miR-542-3p. However, they did not evaluate endogenous miR-542-3p levels in human vitreous fluid. Thus, to our knowledge, our study provides the first data regarding vitreous miR-542-3p expression in PDR. Extensive evidence indicates that miR-542-3p suppresses angiogenesis by directly targeting and negatively regulating Ang-2 expression.[28,29] Moreover, disruption of the NEAT1–miR-542-3p–Ang-2 regulatory axis has been shown to increase Ang-2 expression and exacerbate pathological angiogenesis.[30] Taken together, these results indicate that miR-542-3p may contribute to the post-transcriptional control of Ang-2 and potentially influence angiogenic pathways in ocular vascular disorders. In this context, the non-significant increase in miR-542-3p expression observed in our study may represent a compensatory regulatory response aimed at limiting Ang-2-mediated angiogenic activity underlying PDR pathogenesis.

Regarding miR-5195-3p, a previous study investigating retinal pigment epithelial cell damage showed that miR-5195-3p expression was reduced in retinal epithelial cells exposed to high-glucose conditions compared to controls.[31] However, the expression of miR-5195-3p in the human vitreous remains unexplored in the existing literature. Our miRWalk-based predictions highlight a potential interaction with Ang-2, suggesting that this miRNA acts as a key signaling component in the pathological vascularization characteristic of PDR.

Wang et al.[32] demonstrated that miR-145-5p directly targets Ang-2 and may exert an inhibitory effect on angiogenic signaling. In addition, miR-145-5p has been implicated in ocular angiogenesis, with its expression reported to be upregulated in the retinas of mice with oxygen-induced retinopathy.[33] More recently, miR-145-5p has been shown to contribute to endothelial dysfunction in DR by suppressing PDZK1 under hyperglycemic conditions.[34] Although exosomal miR-145-5p has been detected in the vitreous fluid of patients with pathological

myopia, our study is the first to evaluate vitreous miR-145-5p expression in PDR. In our study, vitreous miR-145-5p expression was decreased in PDR patients compared with controls (fold change: 0.71). Given that Ang-2 is a validated target of miR-145-5p and a key pro-angiogenic mediator in PDR, reduced vitreous miR-145-5p levels may facilitate Ang-2-driven pathological angiogenesis within the vitreous microenvironment. Our findings indicate that dysregulated miR-145-5p expression may contribute to PDR pathogenesis in a compartment-specific manner.

Overall, the results of this study should be considered exploratory and hypothesis-generating. The observed alterations in miRNA expression and angiopoietin levels may reflect complex regulatory processes involved in PDR, but do not establish causal relationships.

Study Limitations

These findings should be interpreted in the context of several limitations. Primarily, the modest sample size may have limited the statistical power to detect significant differences in vitreous miRNA expression, despite the presence of potentially relevant biological effects. Another important limitation of this study is the use of a heterogeneous control group consisting of patients with rhegmatogenous retinal detachment, macular hole, and epiretinal membrane. Although these conditions are not primarily angiogenic, they may alter the vitreous molecular environment and therefore act as potential confounding factors in vitreous biomarker analysis. In addition, multiple comparisons were performed without formal adjustment, which may increase the risk of type I error. The lack of stratification according to disease activity or clinical phenotype (e.g., presence of vitreous hemorrhage or tractional retinal detachment) may further limit the interpretation of the findings. Finally, the cross-sectional design precludes causal inference and limits the ability to assess temporal relationships between miRNA expression and angiogenic alterations.

Accordingly, future investigations with larger patient cohorts are required to confirm these observations and delineate temporal changes in vitreous miRNA expression during disease progression.

Conclusion

The present study demonstrates that differentially expressed miRNAs may be involved in the molecular mechanisms underlying PDR. Our findings suggest that altered vitreous expression of miR-126-3p and miR-204-5p may be associated with angiogenesis-related changes in PDR. However, given the exploratory design, limited sample size, and control group characteristics, particularly the use of a heterogeneous non-healthy control group, these findings should be interpreted cautiously and require confirmation in larger, well-designed studies.

Notably, to the best of our knowledge, this study is the first to evaluate the vitreous expression levels of miR-145-5p, miR-5195-3p, and miR-542-3p in patients with PDR, thereby contributing novel data to the existing literature. Nevertheless, further experimental and clinical studies are warranted to clarify the signaling pathways and functional roles of the significantly dysregulated miRNAs identified in this study.

Ethics

Ethics Committee Approval: All experimental procedures were approved by the Trakya University Faculty of Medicine Scientific Research Ethics Committee (protocol code: TÜTF-BAEK 2020/273, decision no: 14/22, date: 14.09.2020).

Informed Consent: Informed consent was obtained from all participants.

Declarations

Authorship Contributions

Surgical and Medical Practices: İ.A., S.A.Ö., Concept: L.A., Design: L.A., Data Collection or Processing: L.A., H.S., İ.A., S.A.Ö., Analysis or Interpretation: L.A., H.S., İ.A., S.A.Ö., Literature Search: L.A., H.S., Writing: L.A., H.S., İ.A., S.A.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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