Original Article

Serum MicroRNA-126-3p and Serum Vascular Endothelial Growth Factor for Vision Threatening Diabetic Retinopathy in Type 2 Diabetes Mellitus Patients

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ABSTRACT

Purpose: To investigate the expression of miRNA-126-3p and serum VEGF levels in patients with type 2 diabetes Mellitus (DM).

Study Design: Cross-sectional analytical study.

Place and Duration of Study: Prof Ngoerah General Hospital as a tertiary hospital in Bali, Indonesia from January-April 2022.

Methods: This study involved 35 vision-threatening diabetic retinopathy (VTDR) and 30 non-VTDR patients of type 2 DM. After diagnosis of DR by vitreoretinal specialist, blood samples were drawn. Real-time polymerase chain reaction assays were performed to examine miRNA-126-3p and ELISA to detect VEGF. Continuous data were analyzed using median (minimum-maximum range) and categorical data were analyzed using percentages. The statistical analysis was conducted using SPSS for Windows version 25.0.Thedifference and relationship between the proportions were tested by the Chi-square test and logistic regression test.

Results: In case group, there were 23 subjects (65.7%) with low serum miRNA-126-3p expression, while in the control group, there were 7 subjects (23.3%) with low serum miRNA-126-3p expression (OR=6.3; 95% CI 2.1-18.86). In the case group, 20 individuals (57.1%) had high serum VEGF levels of 71.6 ng/L, whereas in the control group, there were 6 individuals (20.0%) with high serum VEGF levels. There was a statistically significant difference between the two groups (OR=5.33; 95% CI 1.75-16.3).

Conclusion: The low serum miRNA-126-3p expression and high serum VEGF levels are associated with vision threatening diabetic retinopathy in patients with type 2 diabetes.

Keywords: Microrna-126-3p, Vascular Endothelial Growth Factor, Diabetic Retinopathy, Diabetes Mellitus.

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INTRODUCTION

One microvascular consequence of diabetes mellitus (DM) that impacts the eyes is diabetic retinopathy (DR). Diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) are included in vision-threatening diabetic retinopathy (VTDR). According to the International Diabetes Federation (IDF), there were 463 million young adults with diabetes mellitus (DM) in 2019, and that figure is expected to rise to 700 million by 2045.[1,2] Moreover, DR is thought to affect one-third of DM patients, and of those who have DR, one-third will develop VTDR. Out of 160 million people with DR in 2019, 47 million

people had VTDR. This number is expected to increase to 242 million DR patients, with 71 million experiencing VTDR, by the year 2045.[1,2]

Type 2 DM accounts for 90-95 percent of DM cases.[3]In 2010, the number of young adults with DM in Indonesia was approximately 6.9 million. It is estimated to rise by 12 million in 2030, with around 43.1% of them experiencing retinopathy complications.[4]

Chronic hypoxia and hyperglycemia conditions among DM individuals activate several pathways of biochemical changes and increase oxidative stress. These conditions result in microvascular damage, particularly in the endothelial cells of retinal blood vessels. This damage serves as a trigger for the progression of diabetic retinopathy into VTDR.[5]

Inflammation and angiogenesis in the retina play a significant role in the development of VTDR, with vascular endothelial growth factor (VEGF) being the main molecule involved.[5] VEGF is responsible for inducing vascular permeability and disrupting the blood-retinal barrier (BRB) in DME, as well as angiogenesis process in PDR. This disruption is caused by ability of VEGF ability to phosphorylate tight junction molecules like occludin and zonular occludens, leading to BRB disruption. An abundance of VEGF in the retina can trigger angiogenesis, resulting in the formation of new blood vessels in retina, which exacerbates the inflammatory process and worsens the conditions of DR.[6,7]

An endogenous sequence known as microRNA (miRNA) inhibits the expression of genes at the post-transcriptional level by means of translational repression. Furthermore, target messenger RNA (mRNA) is also degraded by miRNA. The 3' untranslated region (UTR) of the target mRNA is the precise location where miRNA interacts. Specifically, the majority of vascular endothelial cells have miRNA-126-3p, which is involved in controlling the expression of the VEGF gene and the VEGF signalling pathway.[8]

VTDR in type 2 diabetes mellitus (DM) is a process in which angiogenesis and inflammation are key players, while VEGF also plays a major role. Studies conducted on VEGF serum samples in DR have shown inconsistent results. Similarly, studies conducted at the microRNA level in DR have also been unable to produce a consistent miRNA profile.[8,9] Furthermore, there have been no studies conducted to investigate miRNA-126-3p as a risk factor for VTDR, which consists of PDR and DME groups. This study strengthens the theory regarding the role of miRNA in VTDR conditions by providing evidence that patients with type 2 DM and VTDR have low serum expression of miRNA-126-3p and high serum VEGF levels.

METHODS

This was a case-control study conducted to investigate type 2 DM patients with VTDR as the case group and type 2 DM patients without VTDR as the control group. The research was conducted at Prof. Ngoerah General Hospital and the ethical clearance was granted from the Research Ethics Committee of the Faculty of Medicine Udayana University (No. 63/UN14.2.2.VII.14/LT/2022).

The independent variables (risk factors) were serum miRNA-126-3p expression and serum VEGF levels. The dependent variable (effect) studied was VTDR. Control variables included duration of diabetes mellitus, hypertension, anti-diabetic drugs, and HbA1c levels. The patients' history was obtained, including their name, age, gender, duration of diabetes mellitus, type of anti-diabetic drug and their history of current illnesses such as hypertension, hemodialysis, and malignancy. Additionally, the history of surgery/intravitreal injection was recorded based on the research questionnaire sheet. The diagnosis of VTDR was established through examination by a vitreoretinal specialist. Visual examination was performed using Snellen chart, and the anterior and posterior segments were examined using slit lamp biomicroscopy and 78-lens.

The blood sampling was performed by drawing 3cc of blood from the cubital vein in Ethylenediaminetetraacetic acid (EDTA) tube to analyze the levels of HbA1c. Additionally, 4cc of cubital venous blood was collected in a plain red tube. The plain red tubes were then centrifuged at 3,000 rpm for 15 minutes using the Haraeus TM Labofuge TM 400R centrifuge (ThermoFischer, Waltham, MA, USA), at a temperature of 4°C for 10 minutes. After centrifugation, the samples were inserted into 3 polypropylene tubes and stored at -80°C.

To determine the required sample size, we considered the case proportion (0.60), control proportion (0.25), 95% confidence interval (CI) with a Zα value of 1.96, and a power value of Zβ=80%.

Based on these considerations, the calculated required sample size (N) for both the case and control groups was determined to be 60 subjects.

RNA was extracted from 400 serum samples with a micron size using qT-PCR (Quantitative Real-Time Polymerase Chain Reaction). The mirVana PARIS RNA reagent was used to extract all of the RNA, including miRNA. The TaqMan MicroRNA Reverse Transcription Kit and a TAKARA Thermal Cycler PCR equipment were used to do reverse transcription. Real-time PCR utilizing the TaqMan Fast Advanced Master Mix and TaqMan MicroRNA test reagents was used to detect expression. A reference/calibrator sample and endogenous control gene are needed for the realistic computation of gene expression (comparative delta CT=$2^{-(\Delta\Delta CT)}$).[9]

To standardize the amount of target miRNAs to the total number of miRNAs in the sample, miRNA-328-3p is the endogenous control used in this investigation. For contrast, the sample calibrator is drawn from a group of healthy people. A metric known as Relative Quantification (RQ) represents the number of times the target's miRNA expression in the sample is compared to the target's miRNA expression in the sample calibrator. Subsequently, this number serves as a cut-off point, with readings below it being categorized as low. By utilizing the ROC curve to calculate the statistical magnitude, the cut-off point is ascertained.

Serum VEGF levels were assessed through an enzyme-linked immunosorbent assay (ELISA) technique employing the BT LAB (Bioassay Technology Laboratory) ELISA kit. The results were calculated using computer-based fitting software, and the most accurate fit was determined through regression analysis. The data were then used to define an ELISA cut-off point, with levels over the cut-off point being categorized as high. The statistical magnitude is calculated using the ROC curve to determine the cut-off point.

Patient characteristics were analyzed descriptively and presented in the form of graphs and tables. Continuous data were analyzed using median (minimum-maximum range) and categorical data were analyzed using percentages. The statistical analysis was conducted using SPSS for Windows version 25.0.

ROC analysis was performed to determine the optimal cut-off point for miRNA-126-3p expression and VEGF levels as predictors of VTDR. The area under the curve was evaluated to assess the predictive ability of miRNA-126-3p expression and VEGF levels for VTDR. An area under the curve of 70% or greater indicates a good predictive ability. The best intersection point was determined by identifying the coordinates that were furthest from the diagonal line or closest to coordinates 1.

Chi-square test and odds ratio analysis were conducted. The precision of the data was expressed with a 95% confidence interval (CI) and the level of significance was set at p-value < 0.05. To identify the most important factor in VTDR, multivariate analysis with logistic regression was utilized.

RESULTS

This study is a cross-sectional analytical study aimed at identifying the risk factors for VTDR in individuals with type 2 diabetes. The study involved a total of 65 subjects, comprising 35 VTDR cases and 30 non-VTDR controls selected purposively-consecutively.

Table 1: *Characteristics of Subjects Based on Case and Control Groups.*

Characteristics	Case Group n =35 (100%)	Control Group n =30 (100%)	p-value*
Male	15 (42.9)	13 (43.3)	0.969
Female	20 (57.1)	17 (56.7)	
Age (median(interval)	53 (46-70)	56.5 (45-70)	0.472
Hypertension			
Yes	18 (51.4)	12 (40)	0.357
No	17 (48.6)	18 (60)	
Duration for DM			
≥ 10 years	18 (51.4)	8 (26.7)	0.042
< 10 years	17 (48.6)	22 (73.3)	
Diabetes Medication			0.002*
OHD	12 (34.3)	22 (73.3)	
Insulin	15 (42.9)	3 (10.0)	
OHD + Insulin	7 (20.0)	2 (6.7)	
None	1 (2.9)	3 (10.0)	
HbA1c Level			
High ≥ 7%	26 (74.3)	17 (56.7)	0.135
Low < 7%	9 (25.7)	13 (43.3)	

*Statistically significant if the p-value < 0.05. DM = diabetes mellitus; OHD = oral hypoglycemic drugs; HbA1c = glycated hemoglobin.

Chi-square analysis revealed no statistically significant variations in the control variables between the two groups, with the exception of diabetic

Table 2: *MiRNA-126-3p Serum Expression Distribution in Case and Control Group*.

		Group		OR	CI 95%	p-value
		Case	Control			
miRNA-126-3p Expression	Low(<0.975)	23 (65.7)	7(23.3)	6.3	2.1-18.86	0.001
	High(≥0.975)	12 (34.3)	23(76.7)			

*Statistically significant if the p-value < 0.05.

Table 3: *VEGF Serum Level in Case and Control Group*.

		Group		OR	CI 95%	p-value
		Case	Control			
VEGF serum level	High(≥71.6 ng/L)	20 (57.1)	6 (20.0)	5.33	1.75-16.3	0.002
	Low(<71.6 ng/L)	15 (42.9)	24(80.0)			

*Statistically significant if the p-value < 0.05.VEGF = vascular endothelial growth factor.

medication. In the control group, the majority of individuals used OHD, while in the case group, most were already on insulin. Based on the Mann Whitney test, there were no differences in age between the two groups.

The determination of the cut-off point for miRNA-126-3p expression was based on the area under the curve (AUC) of the receiver operating characteristic (ROC) analysis. The coordinates that were farthest from the diagonal line had a value of 0.975, indicating a sensitivity of 34% and specificity of 23%.

In the case group, there were 23 subjects (65.7%) with low serum miRNA-126-3p expression, while in the control group, there were 7 subjects (23.3%) with low serum miRNA-126-3p expression. There was a statistically significant difference between the two groups (OR=6.3; 95% CI 2.1-18.86). This suggests that individuals with type 2 DM and low serum miRNA-126-3p expression have a six times higher risk of developing VTDR compared to those with high serum miRNA-126-3p expression. The detailed results can be found in Table 2.

ROC analysis was conducted to determine the cut-off point for serum VEGF levels. The coordinates that were farthest from the diagonal line were found to be 71.6 ng/L, with a sensitivity of 57% and specificity of 80%. This result indicates that a serum VEGF level of more than or equal to 71.6 ng/L is considered high.

In the case group, it was observed that 20 individuals (57.1%) had high serum VEGF levels of 71.6 ng/L, whereas in the control group, there were 6 individuals (20.0%) with high serum VEGF levels. There was a statistically significant difference between the two groups (OR=5.33; 95% CI 1.75-16.3). This suggests that the risk of VTDR in individuals with type 2 DM and high serum VEGF levels is five times higher compared to those with low serum VEGF levels.

A multivariate logistic regression analysis with the backward method was conducted to examine correlation between various independent and control variables with VTDR in type 2 DM. These variables included gender, duration of diabetes mellitus, history of hypertension, DM therapy, HbA1c levels, miRNA-126-3p expression, VEGF levels, and IL-1β levels. The analysis revealed that low miRNA-126-3p expression was a significant risk factor for VTDR (OR=61.85; 95% CI 6.33-604.27; p 0.000). Additionally, high serum VEGF levels were found to be a risk factor for VTDR in type 2 DM (OR=53.17; 95% CI 5.37-526.46; p 0.001). High HbA1c levels, as a control variable, were also identified as a risk factor for VTDR in type 2 DM (OR=5.34; 95% CI1.28-22.27; p 0.021).

DISCUSSION

This study evaluates the role of miRNA in VTDR among patients with type 2 DM, in which individuals with low serum expression of miRNA-126-3p and high serum VEGF levels have a higher risk of developing VTDR. This study found that the control group had a higher prevalence of DM duration less than 10 years. Generally, the prevalence of retinopathy is low in DM patients with a duration of less than 10 years, particularly in the non-proliferative DR phase.[10] The periodic screening involving DM patients is crucial due to the significant role of DM duration as a risk factor for DR complications.[6,11]

A longer duration of DM is associated with severity of retinopathy, as it leads to changes in retinal structure and vascularity.[12,13] Chronic exposure to hyperglycemia causes biochemical and vascular changes in retina, including cellular changes in the basement membrane of retinal cells, loss of pericytes in retinal capillaries, retinal basement membrane thickening andcapillariesacellularity.[14]

The oral hypoglycemic drugs (OHD), insulin and a combination of OHD and insulin were used as anti-diabetic drugs in this study. It was found that there were more OHD users compared to insulin, which is consistent with the Riskesdas 2018 data for DM treatment in Bali province. The data showed that 64.6% of DM patients were treated with OHD, 15.7% with insulin, 15.3% with a combination of OHD and insulin and 4.4% received no therapy.[4]

In this study, higher levels of HbA1c were observed in the case group. Through multivariate analysis, we identified high HbA1c levels as a risk factor for VTDR. This result is consistent with previous research by Chatziralli et al and Sasongko et al.[15,16] The target level of HbA1c in DM is commonly under 7%, as reducing HbA1c levels can reduce the occurrence of complications.[11]

To date, there have been no studies investigating the role of serum miRNA-126-3p as a risk factor for VTDR (PDR and DME). However, a study conducted by Qin et al, compared the serum of type 2 diabetes mellitus patients with PDR to a healthy group.[17] A low miRNA-126-3p expression were found in retinal tissue with hypoxia-hyperglycemic conditions. miRNA-126-3p also regulates a number of angiogenic factors that react in hypoxia-associated retinopathy, thereby reducing the process of retinal neovascularization.[18] A study by Bai et al, found that miRNA-126-3p intravitreal injection in a hypoxic retinopathy mouse model could reduce BRB damage through regulation of VCAM-1.[19]

MicroRNAs are easily isolated and have a stable structure; therefore, circulating miRNAs are considered suitable biomarkers for physiological and pathological processes.[17] The changes in the expression of circulating miRNA-126-3p can also provide insight into the likelihood of developing DM in high-risk patients. Serum miRNA-126-3p has potential as a biomarker for screening individuals at risk of pre-diabetes within the healthy population.[20] Research conducted by Rezk et al, demonstrated that in addition to being a diagnostic marker, serum miRNA-126-3p can also be used for monitoring diabetes. This is due to the low expression of serum miRNA-126-3p in DM patients with complications compared to those without complications.[20]

MicroRNA-126-3p is the most abundant miRNA produced in endothelial cells, which plays a crucial role in maintaining vascular structure and promoting angiogenesis. Therefore, miRNA-126-3p has been extensively studied concerning vascular diseases such as coronary artery disease and intracerebral hemorrhage.[21,22] Assessing the level of circulating miRNA-126-3p enables measurement of therapeutic efficacy in treating microvascular damage. Donghui et al, indicated that an increase in miRNA-126-3p levels demonstrated an improvement in microvascular endothelial function after exercise and diet therapy in obese adolescents.[23] However, a study by De la Torre et al, on a population with type 1 diabetes yielded different results, where an increase in miRNA-126-3p expression was observed in the circulation compared to healthy controls, and not significantly different between the DR and non-DR groups.[24]

In this study, elevated levels of VEGF were observed in the group exhibiting more severe DR. This suggests that a rise in retinal VEGF in VTDR patients can be detected in the circulation. This is likely attributed to the defect in existing barrier system between ocular and systemic environment. These circumstances enable serum VEGF to serve as a biomarker for VTDR.

Research has been conducted on miRNA-126-3p in DM and DR groups; however, there has been no investigation into miRNA-126-3p as a risk factor for VTDR. Previous studies have demonstrated that serum levels of miRNA-126-3p were higher in the PDR group compared to the healthy group. This study is the first to examine role of serum miRNA-126-3p in the VTDR group (PDR and DME) in comparison to NPDR and NDR. The role of VEGF as a pro-angiogenesis and pro-inflammatory factor in DR severity has been reported, although the results have been inconsistent. This study demonstrated the role of low miRNA-126-3p in promoting angiogenesis in PDR and increasing retinal vascular permeability in DME among patients with type 2 diabetes and VTDR. Additionally, it established a correlation between microRNA and the angiogenesis-inflammatory processes involved in the pathogenesis of VTDR.

Further research is needed to assess the differences in miRNA-126-3p expression and VEGF levels in serum, vitreous, or aqueous humor. It can also aim to compare the levels of markers in the circulation and the eye as the target organ, specifically concerning other microvascular complications of DM and their role in VTDR. A cohort study can be conducted to determine the role of serum miRNA-126-3p in the progression of DR and the repair of microvascular damage caused by DM therapy.

One limitation of this study is that it did not account for the relationship between variables. All independent variables in this study are considered to be at an equivalent level and are directly related to the VTDR as the dependent variable. Therefore, it is recommended that future research utilize path analysis to thoroughly examine the relationship between variables in the pathogenesis of VPDR.

The findings of this study will provide important information indicating that serum miRNA-126-3p expression can be a risk factor for VTDR. The results of this study can serve as a reference for the treatment of DR, specifically by closely monitoring high-risk groups to prevent blindness caused by DM.

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Patient's Consent: Researchers followed the guidelines set forth in the Declaration of Helsinki.

Conflict of Interest: Authors declared no conflict of interest.

Ethical Approval: The study was approved by the Institutional review board/Ethical review board **(63/UN14.2.2.VII.14/LT/2022)**.

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Authors Designation and Contribution

Ni Made Ayu Surasmiati; Medical Staff: *Concepts, Design, Literature search, Data acquisition, Data analysis, Manuscript preparation, Manuscript editing, Manuscript review.*

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