European Review for Medical and Pharmacological Sciences 2019; 23: 6387-6393

Expression and significance of MiR-126 and VEGF in proliferative diabetic retinopathy

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Abstract. – OBJECTIVE: To study the expression and significance of micro ribonucleic acid (miR)-126 and vascular endothelial growth factor (VEGF) in the vitreous body and proliferative membrane tissues of affected eyes and plasma in proliferative diabetic retinopathy (PDR).

PATIENTS AND METHODS: A total of 50 PDR patients admitted to our hospital from January 2017 to January 2018 were collected, including 17 cases in stage IV, 20 cases in stage V, and 13 cases in stage VI according to the DR staging criteria. Another 30 patients with an idiopathic macular hole were selected as control group. After admission, the examinations were performed, venous blood was drawn, and vitrectomy was performed, during which the vitreous body and proliferative membrane tissues were taken. The plasma VEGF content was detected via enzyme-linked immunosorbent assay (ELISA). The VEGF protein expression level was detected via Western blotting in vitreous body and proliferative membrane tissues. Moreover, the miR-126 expression was detected via quantitative polymerase chain reaction (qPCR).

RESULTS: In the vitreous body tissues, proliferative membrane tissues and plasma, the expression level of miR-126 was significantly lower in control group than that in stage IV, V, and VI groups ($p<0.05$). The miR-126 expression was significantly lower in stage IV group than that in stage V and VI groups ($p<0.05$), meanwhile, miR-126 expression was significantly lower in stage V group than that in stage VI group ($p<0.05$). In the vitreous body tissues, proliferative membrane tissues and plasma, the VEGF mRNA and protein expression levels were significantly higher in control group than those in stage IV, V, and VI groups ($p<0.05$). The VEGF expressions were significantly higher in stage IV group than those in stage V and VI groups ($p<0.05$). In addition, VEGF expressions were significantly higher in stage V group than those in stage VI group ($p<0.05$). Furthermore, the VEGF expression level was negatively correlated with the miR-126 expression level.

CONCLUSIONS: We found that the abnormally high expression of miR-126 negatively regulates VEGF, which may be one of the important mechanisms of occurrence of PDR. The miR-126 and VEGF content can serve as important predictors for the condition of PDR.

Key Words:
MiR-126, VEGF, Proliferative diabetic retinopathy.

Introduction

Type 2 diabetes mellitus (T2DM) is the most common glucose metabolic disorder, which can lead to various severe complications. Proliferative diabetic retinopathy (PDR) is one of those complications. Besides, PDR can cause a diminution of vision and even blindness[1]. The pathogenesis of PDR is complex and remains unclear yet. However, it is currently known that the incidence of PDR is closely related to the endocrine and metabolic disorders in T2DM patients. The inflammation, excessive oxygen free radical reaction, activation of the diacylglycerol-protein kinase C system, and accumulation of advanced glycation end products are considered to be correlated with the incidence of PDR.

The micro-ribonucleic acid (miRNA) is a kind of non-coding RNA composed of about 18-25 nucleotides. MiRNAs can bind to the 3'untranslated region of the target RNA, thus inhibiting the translation of the target RNA[2-5]. In the miRNA family, miR-126 is mainly expressed in endothelial cells and located in the epidermal growth factor-like domain 7, thereby exerting an important regulatory effect on its transcription[6,7]. Currently, it is believed that miR-126 is involved in many important physiological processes, such as cell migration and gland development. Some studies

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showed that miR-126 could regulate the vascular endothelial growth factor (VEGF) signaling pathway. In addition, others have demonstrated that the miR-126 expression is down-regulated in new retinal vessels. Furthermore, some scholars[8,9] have found that miR-126 possesses an important promoting effect on angiogenesis and a potent regulatory effect on VEGF, which can inhibit the VEGF expression, suppressing the formation of new vessels. Therefore, miR-126 plays an important regulatory role in the retinal vascular endothelial function and retinal angiogenesis, which is considered a potentially effective target for the treatment of retinal diseases.

However, the expression and clinical significance of miR-126 and VEGF in the vitreous body and proliferative membrane tissues of affected eyes and plasma in PDR patients are still unclear. Therefore, this study aims to elucidate the expressions of miR-126 and VEGF in the vitreous body and proliferative membrane tissues of affected eyes and plasma in PDR, and summarize their guiding significance in the clinical diagnosis and treatment of PDR.

Patients and Methods

General Data

A total of 50 PDR patients admitted to our hospital from January 2017 to January 2018 were collected, including 17 cases in stage IV, 20 cases in stage V, and 13 cases in stage VI according to the DR staging criteria. Another 30 patients with idiopathic macular hole (IMH) were selected as the control group. All patients underwent the elective vitrectomy. There were 8 males and 9 females with an average age of (55.83±5.69) years old in stage IV group, 12 males and 8 females with an average age of (57.12±4.18) years old in stage V group, and 7 males and 6 females with an average age of (54.39±6.63) years old in stage VI group. In control group, there were 16 males and 14 females with an average age of (53.33±7.21) years old. Inclusion criteria for PDR: (1) PDR caused by T2DM, (2) repeated vitreous hemorrhage, and pre-retinal membrane formed shown in color Doppler ultrasonography without drug therapy, and (3) the eye examination displayed the formation of fundus proliferative membrane or tractional detachment of the retina. All patients signed the informed consent and agreed to participate in the investigation. This study was approved by the Ethics Committee of Tangshan Worker' Hospital.

Reagents and Instruments

Anti-VEGF antibody (Abcam, Cambridge, MA, USA), enzyme-linked immunosorbent assay (ELISA) kit (Maxim, Fuzhou, China), AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit (Vazyme, Nanjing, China), HiScript II Q RT SμperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), optical microscope (Leica DMI 4000B/DFC425C, Solms, Germany), fluorescence qPCR instrument (ABI 7500, Foster City, CA, USA), Image-Lab analysis system and Image-Pro analysis system (Bio-Rad, Hercules, CA, USA).

Research Methods

After admission, the examinations were performed for PDR patients and IMH patients meeting the inclusion criteria, venous blood was collected and vitrectomy was performed under anesthesia. 0.5 mL vitreous body was taken using a vitreous tip and stored in a centrifuge tube. The pre-retinal proliferative membrane was taken during operation in observation group, while the internal limiting membrane tissues were taken during operation in control group, and stored in the centrifuge tube at -80°C.

ELISA

The plasma VEGF content was detected *via* ELISA using the following method: the venous blood collected was treated according to the instructions of the ELISA kit, and then, the plasma VEGF content was detected in accordance with the instructions of the ELISA kit.

Western Blotting

The bone tissues stored at -20°C were added with the lysis buffer and centrifuged. The protein was quantified using bicinchoninic acid (BCA; Pierce, Rockford, IL, USA), and the protein concentration was measured. After denaturation, the protein was separated *via* gel electrophoresis, transferred onto a membrane, sealed for 1.5 h and incubated with anti-VEGF primary antibody (1:1000) and secondary antibody (1:1000). After the membrane was washed with Tris-Buffered Saline & Tween (TBST) to remove the secondary antibody, the image was developed. Then, the membrane was placed in the chemiluminescence reagent for reaction for 1 min, followed by image development in a dark place and analysis using the gel scanning imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

Table I. Primer sequences.

Gene	Primer sequence
miR-126	Forward: 5'-UCGUACCGUGAGUAAUAAUGC-3' Reverse: 5'-CACTTCCTCAGCACTTGTTGGTAT-3'
VEGF	Forward: 5'-TGCCCACTGAGGAGTCCAAC-3' Reverse: 5'-TGGTTCCCGAAACGCTGAG-3'
GAPDH	Forward: 5'-ACGGCAAGTTCAACGGCACAG-3' Reverse: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

qPCR

The total RNA was extracted from tissues and plasma using the RNA extraction kit, and reversely transcribed into complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit. The reaction system was 20 μL, and the reaction conditions are as follows: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, annealing at 60°C for 30 s, a total of 40 cycles. The relative expression level of miR-126 was calculated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences were shown in Table I.

Statistical Analysis

In this study, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Enumeration data were expressed as mean ± standard deviation. The *t*-test was used for the data in line with normal distribution and homogeneity of variance, corrected *t*-test for the data in line with normal distribution and heterogeneity of variance, and non-parametric test for the data not in line with normal distribution and homogeneity of variance. Rank sum test was adopted for ranked data, and chi-square test was adopted for enumeration data. *p*-values < 0.05 were considered statistically significant.

Results

Expression of MiR-126 and VEGF in Vitreous Body Tissues

As shown in Figure 1, the miR-126 content was the highest and the VEGF mRNA content was the lowest in vitreous body tissues in stage VI group. The miR-126 content was the lowest and the VEGF mRNA content was the highest in vitreous body tissues in control group. The miR-126 content was significantly increased, while the VEGF mRNA content was significantly decreased in stage IV, V, and VI groups compared with those in control group, and the differences were statistically significant ($p<0.05$). Compared with those in stage IV group, the miR-126 content was significantly increased, while the VEGF mRNA content was significantly decreased in stage V and VI groups, and the differences were statistically significant ($p<0.05$). Compared with those in stage V group, the miR-126 content was also significantly increased, while the VEGF mRNA content was also significantly decreased in stage VI group, and there were statistically significant differences ($p<0.05$). Moreover, as shown in Figure 2, the VEGF protein content in vitreous body tissues was the lowest in stage VI group and the

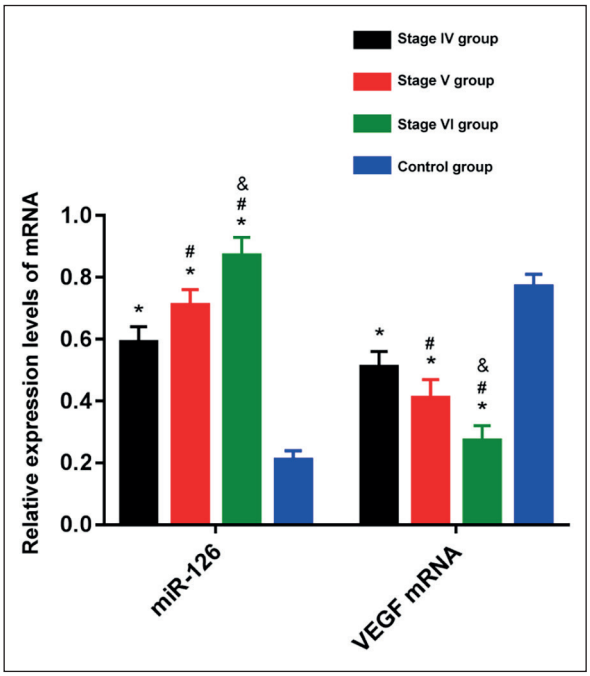

Figure 1. Relative mRNA expression levels of miR-126 and VEGF in vitreous body tissues. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

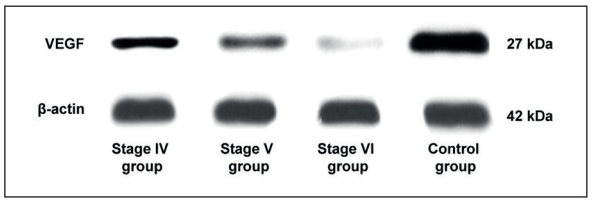

Figure 2. VEGF protein expression in vitreous body tissues detected *via* Western blotting.

highest in control group. The relative expression level of protein is shown in Figure 3. Compared with that in control group, the VEGF protein content in stage IV, V, and VI groups significantly declined, and there were statistically significant differences ($p<0.05$). Compared with that in stage IV group, the VEGF protein content in stage V and VI groups markedly declined, displaying statistically significant differences ($p<0.05$). Compared with that in stage V group, the VEGF protein content in stage VI group was significantly decreased, showing a statistically significant difference ($p<0.05$).

Expression of MiR-126 and VEGF in Proliferative Membrane Tissues

As shown in Figure 4, the miR-126 content was the highest and the VEGF mRNA content was the lowest in proliferative membrane tissues in stage VI group. The miR-126 content was the lowest and the VEGF mRNA content was the highest in proliferative membrane tissues in control group. The miR-126 content was significantly increased, while the VEGF mRNA content was significantly

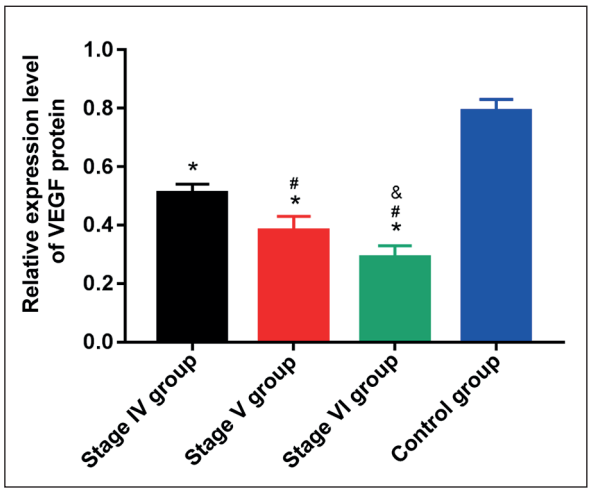

Figure 3. Relative protein expression level of VEGF in vitreous body tissues. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

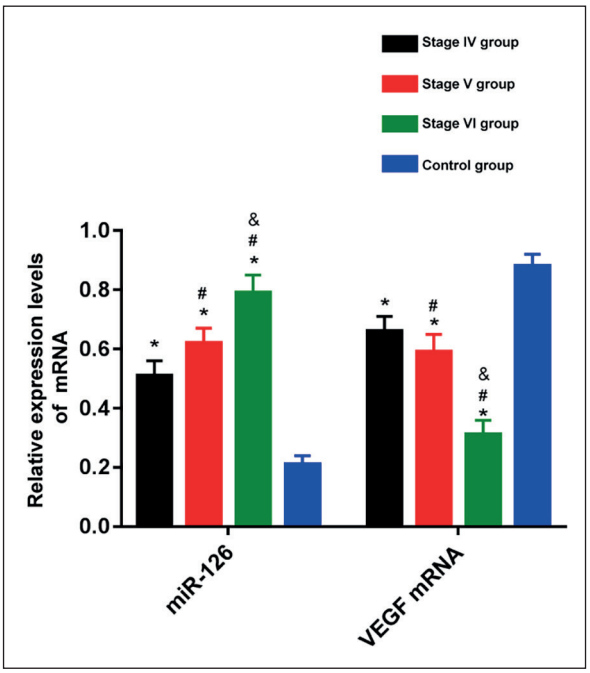

Figure 4. Relative mRNA expression levels of miR-126 and VEGF in proliferative membrane tissues. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

decreased in stage IV, V, and VI groups compared with those in control group, and the differences were statistically significant ($p<0.05$). Compared with those in stage IV group, the miR-126 content was significantly increased, while the VEGF mRNA content was significantly decreased in stage V and VI groups, and the differences were statistically significant ($p<0.05$). Compared with those in stage V group, the miR-126 content was also significantly increased, while the VEGF mRNA content was also significantly decreased in stage VI group, and there were statistically significant differences ($p<0.05$). Moreover, as shown in Figure 5, the VEGF protein content in proliferative membrane tissues was the lowest in stage VI group and the highest in control group. The relative expression level of protein is

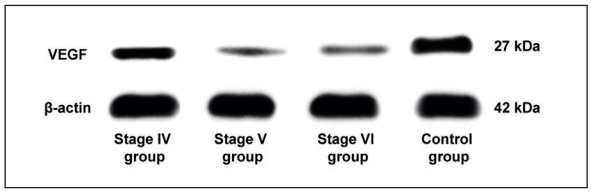

Figure 5. The protein expression of VEGF in proliferative membrane tissues detected *via* Western blotting.

shown in Figure 6. Compared with that in control group, the VEGF protein content in stage IV, V, and VI groups significantly declined, and there were statistically significant differences ($p<0.05$). Compared with that in stage IV group, the VEGF protein content in stage V and VI groups markedly declined, displaying statistically significant differences ($p<0.05$). Compared with that in stage V group, the VEGF protein content in stage VI group was significantly decreased, showing a statistically significant difference ($p<0.05$).

Expression of MiR-126 and VEGF in Plasma

As shown in Figure 7, the miR-126 content was the highest and the VEGF mRNA content was the lowest in plasma in stage VI group. The miR-126 content was the lowest and the VEGF mRNA content was the highest in plasma of control group. The miR-126 content was significantly increased, while the VEGF mRNA content was significantly decreased in stage IV, V, and VI groups compared with those in control group, and the differences were statistically significant ($p<0.05$). Compared with those in stage IV group, the miR-126 content was significantly increased, while the VEGF mRNA content was significantly decreased in stage V and VI groups, and the differences were statistically significant ($p<0.05$). Compared with those in stage V group, the miR-126 content was also significantly increased, while the VEGF mRNA content was also significantly decreased in stage VI group, and there were statistically significant differences ($p<0.05$). Moreover, as shown in Figure 8, the VEGF protein content in plasma was the lowest in stage VI group and the highest in control group. Compared with that in control group, the VEGF protein content in stage IV, V, and VI groups significantly declined, and there were statistically significant differences ($p<0.05$). Compared with that in stage IV group, the VEGF protein content in stage V and VI groups mark-

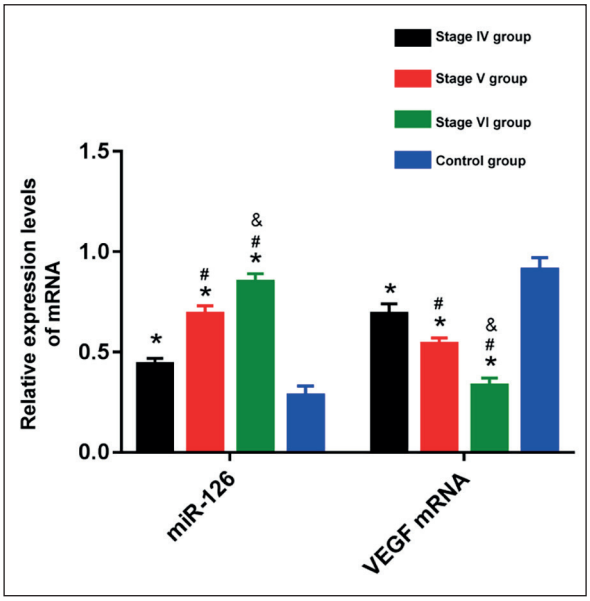

Figure 7. Relative mRNA expression levels of miR-126 and VEGF in plasma. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

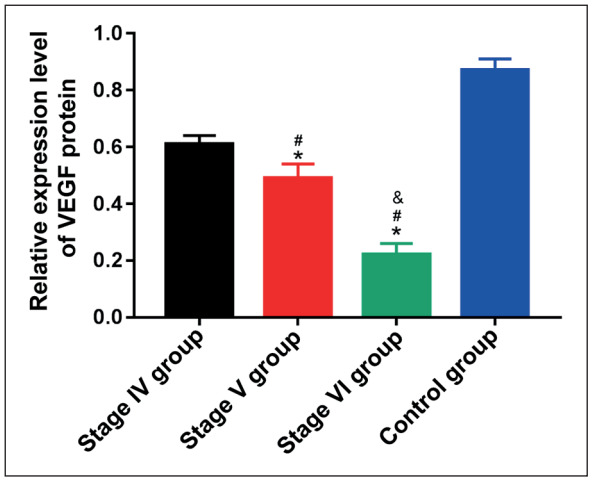

Figure 6. Relative protein expression level of VEGF in proliferative membrane tissues. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

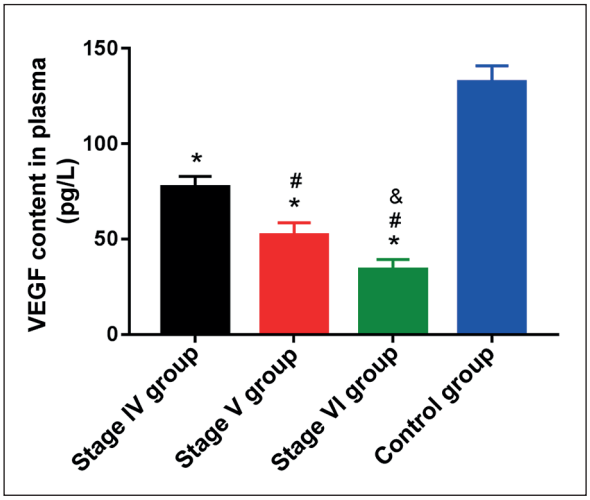

Figure 8. VEGF content in plasma detected *via* ELISA. Note: $p^{*}<0.05$ *vs.* control group, $p^{\#}<0.05$ *vs.* stage IV group, $p^{\&}<0.05$ *vs.* stage V group.

edly declined, displaying statistically significant differences ($p<0.05$). Compared with that in stage V group, the VEGF protein content in stage VI group was significantly decreased, showing a statistically significant difference ($p<0.05$).

Correlation Analysis Between MiR-126 Content and VEGF Expression

The VEGF expression level was negatively correlated with the miR-126 expression level (r = -0.719) (Figure 9).

Discussion

PDR is one of the most common complications of T2DM, whose pathogenesis is complex. Currently, it is believed that the long-term hyperglycemia and poor control effect are the major causes. Hyperglycemia can lead to the poor local blood supply in the retina and pathological responses of ischemia and hypoxia, causing locally high expression of inflammatory factors and inflammation, and affecting angiogenesis in the damaged retina[10,11]. Conversely, there are markedly abnormal retinal angiogenesis and abnormal function, and easy rupture and hemorrhage in the vitreous body of PDR patients, which are considered as the main pathological responses of PDR[12-14]. It is currently believed that VEGF, as the strongest pro-angiogenesis factor, can regulate vascular growth and regeneration. VEGF is also closely correlated with the occurrence, development, and prognosis of DR. Increasing evidence has revealed that miR-126 is involved in regulating genes closely related to angiogenesis and inflammation. Authors[15,16] have demonstrated that miR-126 is involved in the physiological process of vascular development through regulating VEGF to target PI3 kinase and MAP kinase signals. According to further studies[17,18], embryonic vascular dysplasia can occur after the miR-126 knockout, including intracranial hemorrhage and vascular rupture. MiR-126 exerts an important regulatory effect on vascular growth, development, and regeneration through regulating the VEGF signaling pathway. So we speculated that miR-126 is closely related to the pathogenesis and progression of PDR. The results of this work detected that miR-126 was highly expressed in vitreous body tissues and proliferative membrane tissues of affected eyes and plasma of PDR patients, and it was significantly increased compared with that in non-PDR patients. Moreover, the miR-126 expression level in vitreous body tissues and proliferative membrane tissues of patients in stage VI was significantly higher than that of patients in stage V and IV. In other words, the miR-126 expression level rises with the increase of severity of PDR. All those results suggested that there is an abnormally high expression of miR-126 in the body of PDR patients, which may be a potential mechanism of occurrence of PDR.

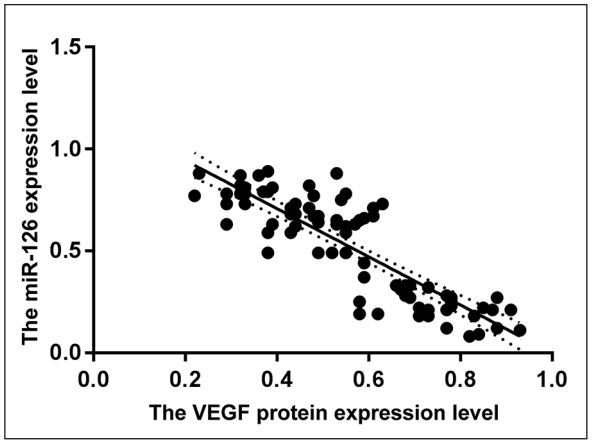

Figure 9. Correlation between VEGF protein and miR-126 expression.

VEGF, as an important angiogenesis promoter, plays an important role in angiogenesis[19]. Studies have shown that the VEGF expression in the retina has its own uniqueness, namely transient characteristic. The VEGF expression is high in the development stage of retinal vessels, and it quickly declines when retinal vessels are formed[20]. The results of this study showed that the VEGF expression level was lower in the vitreous body, proliferative membrane tissues, and plasma of PDR patients. Those results indicated that dysfunction occurs in the retinal angiogenesis of PDR patients, and new vessels cannot be formed in time to maintain the normal physiological needs of the retina. This finding may be one of the key factors for the occurrence of PDR. At the same time, the VEGF protein expression level in vitreous body tissues and proliferative membrane tissues of patients in stage VI was significantly lower than that of patients in stage V and IV. Those results suggested that the VEGF expression in patients with PDR declines with the increase of severity of PDR, and the VEGF expression was negatively correlated with the miR-126 expression. In view of the abnormal expression of miR-126 in PDR patients and the regulatory effect of miR-126 on VEGF, it is

speculated that the abnormally high expression of miR-126 in PDR patients negatively regulates VEGF. This finding may be another important mechanism of PDR occurrence. Thus, the miR-126 and VEGF content can serve as important predictors for the state of PDR.

Conclusions

We found that the abnormally high expression of miR-126 negatively regulates VEGF, which may be one of the important mechanisms of occurrence of PDR. The miR-126 and VEGF content can serve as important predictors for the state of PDR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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