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Roles of Hypoxia-Inducible Factor-1α And Vascular Endothelial Growth Factor in Neovascularization Upon Proliferative Diabetic Retinopathy

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ABSTRACT

Objective: To observe the expressions of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in fibrovascular membrane from patients with proliferative diabetic retinopathy (PDR) and epiretinal membrane from non-diabetic patients with idiopathic macular epiretinal membrane, and to preliminarily explore the roles of HIF-1α and VEGF in neovascularization in the case of PDR.

Methods: A total of 60 patients (60 eyes) diagnosed as type 2 PDR in our hospital from September 2018 to September 2019, who needed to receive 23G pars plana vitrectomy (PPV), were included in this study, and 20 patients diagnosed as macular hole were selected as controls. Experimental group was further divided into two subgroups, i.e. group A (n=30, non-injected PDR group, directly treated with vitrectomy) and group B (n=30, injected PDR group, intravitreally injected with anti-VEGF drug ranibizumab before operation). Pathological specimens of the fibrovascular membrane in PDR and macular epiretinal membrane were obtained during PPV. Immunohistochemical staining and reverse transcription-polymerase chain reaction (RT-PCR) were applied to detect the expressions of HIF-1α, VEGF and VEGF receptor 2 (VEGFR2), and the correlation between two variables was analyzed using Spearman's method.

Results: Immunohistochemical staining showed that the protein expressions of HIF-1α, VEGF and VEGFR2 in the fibrovascular membrane were all positive in experimental group, and they were significantly higher in injected PDR group than those in non-injected PDR group ($P<0.05$). RT-PCR exhibited that the mRNA expressions of HIF-1α and VEGF were the highest in non-injected PDR group and the lowest in control group. There were statistically significant differences in the relative mRNA expression levels of HIF-1α and VEGF in the pathological specimens of fibrovascular membrane and macular epiretinal membrane among control group, injected PDR group and non-injected PDR group ($P<0.05$) as well as between injected PDR group and non-injected PDR group ($P<0.05$). Spearman's analysis revealed that the expression of VEGFR2 was significantly positively correlated with those of HIF-1α and VEGF ($P<0.05$).

Conclusion: HIF-1α, VEGF and VEGFR2 are highly expressed in the fibrovascular membrane from PDR patients, and the VEGF signaling pathway may participate in the formation and development of new vessels in the case of PDR.

KEYWORDS: hypoxia-inducible factor-1α; vascular endothelial growth factor; neovascularization; proliferative diabetic retinopathy

INTRODUCTION

Diabetes mellitus (DM) is a type of chronic metabolic disease characterized by hyperglycemia, which is mainly caused by deficiency or impaired biological effect of insulin *in vivo*, and long-term hyperglycemia will damage various tissues such as cardiovascular system, nerve, kidney and eye [1]. The occurrence of DM complications not only seriously reduces patient's quality of life but also increases social and economic burdens. Diabetic retinopathy (DR) is a DM-induced common and severe microvascular complication, whose major pathological change is retinal microcirculation disturbance, with irreversible visual loss as the main

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manifestation [2]. About 93 million people suffer from DR in the world, 17 million of whom have proliferative DR (PDR) [3]. In China, retinopathy emerges in approximately 24.7-37.5% of DM patients, and PDR accounts for 3.3-7.4% [4], so the risk of visual loss will rise constantly. In the case of advanced PDR, a kind of fundus lesion, many retinal injuries are attributable to retinal neovascularization. The fibrovascular membrane formed by neovascularization and fibrosis imposes traction on the retina to induce serious vision loss [5]. Young PDR patients have a prominently higher risk of blindness than elderly PDR patients because they have rapidly progressing vitreoretinopathy, severer fibrovascular proliferation and wider traction retinal detachment [6].

Hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) are important players in the occurrence and development of PDR [7]. HIF-1α is a key factor in the process of neovascularization. The ubiquitylation and degradation of HIF-1α are decreased under normoxic conditions, while it accumulates and exists stably under hypoxic conditions, thus participating in diverse reactions in cells [8-10]. As a crucial risk factor promoting neovascularization in PDR, VEGF directly binds to vascular endothelial cells to facilitate its pathological proliferation and can induce the secretion of other inflammatory factors, so it is one of the pivotal factors that cause continuous progression of PDR in patients [11]. Based on the above findings, clinical surgical specimens of PDR retinas were subjected to immunohistochemical staining, and the expressions of HIF-1α and VEGF in epiretinal membranes were determined by reverse transcription-polymerase chain reaction (RT-PCR) in this study, so as to investigate the effects of HIF-1α and VEGF on neovascularization in PDR, thereby providing theoretical references for clinical treatment of PDR patients.

MATERIALS AND METHODS

Subjects

Patients diagnosed with type 2 PDR in our hospital from September 2018 to September 2019 and treated with 23G pars plana vitrectomy (PPV) were selected as the subjects. DM patients complicated with other types of retinopathy or patients with a past history of other intraocular surgeries were excluded. A total of 60 PDR patients (60 eyes) aged 26-63 years old and (46.98 ± 5.19) years old on average were included in this study. Then the 60 subjects meeting the diagnostic criteria of PDR [4] were set as experimental group, including 28 males and 32 females, with a course of DM of more than 10 years. Moreover, 20 patients diagnosed as macular hole were enrolled as control group, including 8 males and 12 females. None of the patients had medical history or family history of DM or were accompanied with autoimmune disease, hypertension, severe cardiovascular and cerebrovascular disease or malignant tumor. This study was reviewed by the Ethics Review Committee of the hospital, and the subjects and their families were informed of and signed the informed consent.

Grouping of subjects

The 60 patients in experimental group were randomized into two subgroups. In group A (n=30), patients received intravitreal injection of anti-VEGF drug ranibizumab before operation and vitrectomy at 1 week after drug injection. Patients in group B (n=30) directly underwent vitrectomy. If lens opacity occurred and affected the visual field of patients during operation, phacoemulsification and intraocular lens implantation would be applied in combination.

Sample collection

After topical anesthesia or retrobulbar anesthesia, the eyelid was kept open using an eye speculum, povidone iodine was dropped into the eye routinely, and the conjunctival sac was flushed by salt water. Pathological specimens of fibrovascular membrane in PDR and macular epiretinal membrane were obtained during PPV by means of retinal scissors and retinal forceps. The pathological specimens collected were immediately fixed in 4% paraformaldehyde solution, routinely dehydrated with alcohol, transparentized using xylene and embedded in paraffin, followed by preparation of serial sections (3 μm).

Main reagents and apparatus

Phosphate-buffered saline containing 4% paraformaldehyde (Shanghai Experimental Reagent Co., Ltd.), antigen retrieval buffer, normal goat serum blocking buffer, horseradish peroxidase-labeled avidin working solution and DBA coloration kit (Wuhan Boster Biological Technology Co., Ltd.), hematoxylin (Beijing Solarbio Science & Technology Co., Ltd.), RNAiso Plus, RT kit and fluorescence quantitative PCR kit (TaKaRa, Japan) and primers (Beijing Liuhe BGI) were applied.

A Constellation vitrectomy machine was purchased from Alcon (USA), a fluorescence quantitative RT-PCR system was provided by Roche Diagnostics GmbH, and a high-speed centrifuge was

bought from Beckman (USA). A microcentrifuge was offered by TIANGEN Biotech (Beijing) Co., Ltd., nucleic acid and protein analyzer and transcriptase incubator were purchased from Thermo Fisher Scientific Inc., and a light microscope was provided by Leica (Germany).

HE staining

Right lung tissues of rats were fixed in 4% paraformaldehyde solution for 24 h, then dehydrated with alcohol, embedded in paraffin and stained with hematoxylin-eosin (HE), followed by slicing into 5 μm-thick sections. Finally, the morphological changes in the lung tissues were observed under the light microscope.

Immunohistochemical staining

After baking at 60°C for 1 h, tissue sections (3 μm) were deparaffinized in xylene, dehydrated by gradient ethanol and added with 3% hydrogen peroxide-methanol in drops to remove endogenous peroxidase. Next, antigen retrieval buffer and goat serum blocking buffer were added dropwise for incubation. Subsequently, diluted primary antibodies, biotin-labeled secondary antibodies and horseradish peroxidase-labeled avidin were added in drops sequentially. Finally, the sections were subjected to HE staining, mounted in neutral resin and observed under a light microscope.

Counting of endothelial cells

HE staining was performed for 5 serial sections made from the maximum sectional plane of every tissue. Then the sections were observed under a high-power microscope, and 5 fields of vision were randomly selected from each section to count the vascular endothelial cells. The count of endothelial cells in each specimen under the high-power microscope referred to the average value of the cell count in the 5 fields of vision. Finally, the average value of vascular endothelial cell count in every section in each group was calculated.

RT-PCR

RT-PCR was conducted to measure the messenger ribonucleic acid (mRNA) expressions of HIF-1α, VEGF and VEGF receptor 2 (VEGFR2) in each group of pathological specimens. Specifically, RNAiso Plus was added into retinal tissues (1 mL: 50-100 mg), placed at room temperature for 20 min to fully lyse the tissues. Later, total RNAs were extracted, their optical density (OD) at 260 nm and 280 nm in different samples was detected using an ultraviolet spectrophotometer, and the sample purity was calculated. The samples with an OD_{260}/OD_{280} of 1.7-2.1 were used in the RT-PCR system. With glyceraldehyde-3-phosphate dehydrogenase as an internal reference gene, the gene sequences of HIF-1α, VEGF and VEGFR2 in human were searched from PUBMED/Nucleotide GenBank, primers needed were amplified through Primer Express 5.0 software, and the specificity of target genes was analyzed *via* BLAST (www.ncbi.nlm.nih.gov/BLAST). Primer sequences were designed as follows. HIF-1α: F (5'-CCGATGGAAGCACTAGACA-3'), R (5'-CAAAGCGACAGATAACACG-3'); VEGF: F (5'-ACGGACAGACAGACAGACA-3'), R (5'-CAAGGCAAGGCTCCAATG-3'); VEGFR2: F (5'-AGTGATCGGAAATGACACTG-GA-3'), R (5'-GCACAAAGTGACACGTTGAGAT-3'); GAPDH: F: (5'-CACGATGGAGG-GGCCGGACTCATC-3'), R (5'-TAAAGACCTCTATGCCAACACAGT-3').

Statistical analysis

SPSS 20.0 software was employed for statistical analysis. One-way analysis of variance was used for the expressions of related factors in multiple groups, and Spearman's analysis was conducted for the correlations between two variables. $\alpha=0.05$ and $P<0.05$ indicated statistically significant differences.

RESULTS

Baseline clinical data

The basic clinical data were compared between PDR patients and controls. It was shown that there were no statistical differences in the gender, age and body mass index (BMI) between experimental group (including injected PDR group and non-injected PDR group) and control group ($P>0.05$). Besides, no statistical differences in DR history, PDR history and glycosylated hemoglobin (HbAlc) level were observed between injected PDR group and non-injected PDR group ($P>0.05$) (Table 1).

HE staining results

According to HE staining for morphological observation of membrane specimens, PDR patients had diversified forms of fibrovascular membrane, visible new vessels as well as fibroblast-like or epithelioid fibrocytes and proliferating cells, with occasionally visible lymphocytes and neutrophils. Moreover, new vessels of different numbers and shapes could be seen in the fibrovascular membrane in PDR. Among the 30 patients in injected PDR group, the vessel lumens were relatively narrow, lumen occlusion even occurred in some specimens, and the vascular endothelial cell count was (18.48 ± 2.17) in high-power fields (400×). Dilated vessel lumen and intact morphology

were observed among the 30 patients in non-injected PDR group, with vascular endothelial cell count of (45.15 ± 3.75). However, there were no vascular endothelial cells in macular epiretinal membrane in control group, and there were statistically significant differences between the two experimental groups (P<0.05) (Figure 1).

Table 1. **Baseline clinical data**

Index	Control group	Injected PDR group	Non-injected PDR group	*p*
n	20	30	30	-
Gender (male/female)	8/12	15/15	14/16	0.69
Age (year)	52.6±7.22	55.1±8.5	53.1±8.46	0.43
Course of DM (year)	0	10.1±7.6	11.2±4.6	0.65
Course of PDR (month)	0	12.0±3.7	14.5±1.2	0.38
BMI (kg/m^2)	26.1±3.4	26.4±4.0	26.3±3.6	0.41
HbAlc (%)	5.22±0.41	7.8±1.9	9.0±2.3	0.97

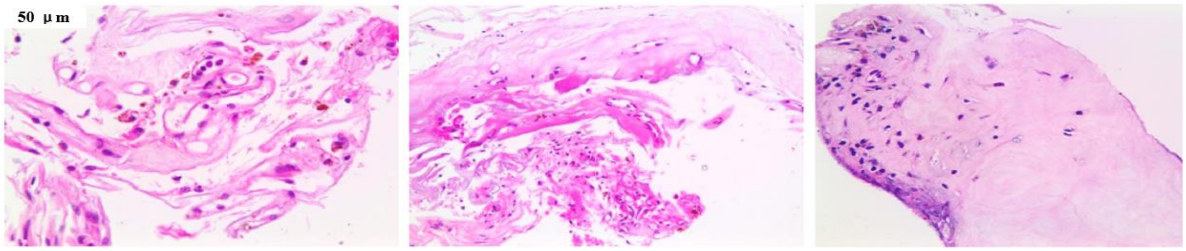

Figure 1**. HE staining results of membrane specimens (400×).**

Immunohistochemical staining results

The results of immunohistochemistry indicated that in the fibrovascular membrane in injected PDR group, there were 26 (86.67%) cases of positive HIF-1α expression, 29 (96.67%) cases of positive VEGF expression and 30 (100%) cases of positive VEGFR2 expression. In addition, the expressions of HIF-1α, VEGF and VEGFR2 were all positive in the fibrovascular membrane in non-injected PDR group. The differences in the protein expressions of HIF-1α, VEGF and VEGFR2 in new vascular endothelia were statistically significant between injected PDR group and non-injected PDR group (P<0.05) (Figure 2 and 3).

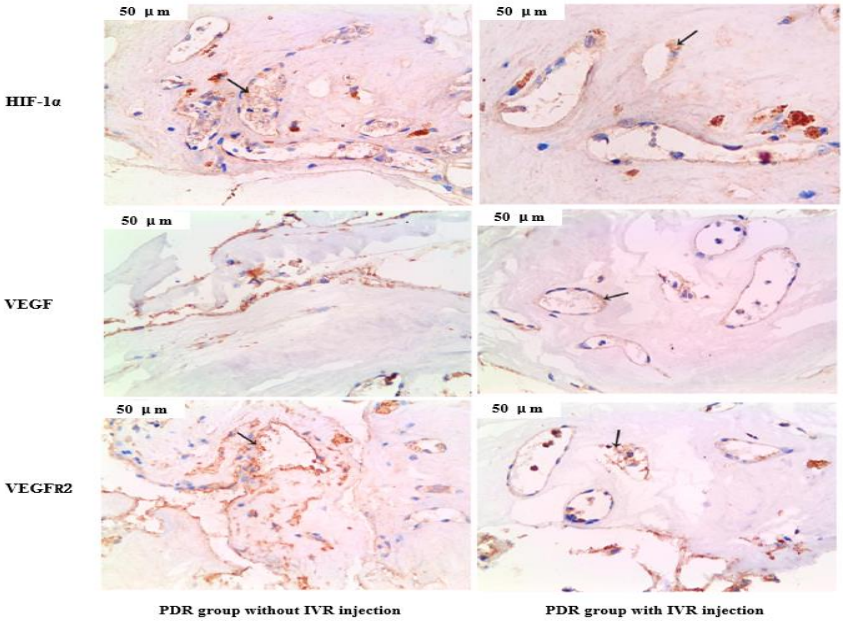

Figure 2**. Immunohistochemical staining results of pathological specimens of ocular fibrovascular membrane from PDR patients (400×).**

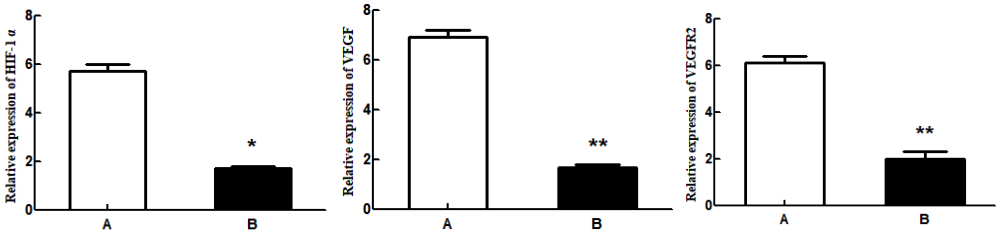

Figure 3. **Relative protein expressions of HIF-1α, VEGF and VEGFR2 in ocular fibrovascular membrane in injected PDR group and non-injected PDR group. A: Non-injected PDR group; B: Injected PDR group. *P<0.05 *vs.* injected PDR group, and **P<0.01 *vs.* injected PDR group.**

RT-PCR results

The expression levels of HIF-1α, VEGF and VEGFR2 in fibrovascular membrane from PDR patients and macular epiretinal membrane from non-diabetic patients with idiopathic macular epiretinal membrane were examined using fluorescence quantitative PCR. It was revealed in the results that the mRNA expressions of HIF-1α, VEGF and VEGFR2 were the highest in non-injected group and the lowest in control group. Besides, there were statistically significant differences in the relative mRNA expression levels of HIF-1α, VEGF and VEGFR2 in the pathological specimens of fibrovascular membrane and macular epiretinal membrane among control group, injected PDR group and non-injected PDR group (P<0.05) as well as between injected PDR group and non-injected PDR group (P<0.05) (Figure 4).

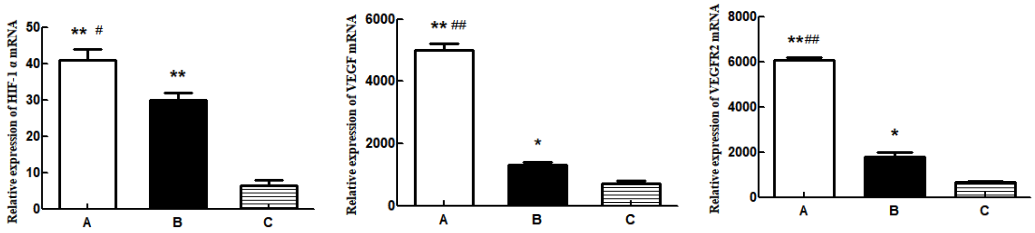

Figure 4**. Relative mRNA expressions of HIF-1α, VEGF and VEGFR2 in each group of pathological specimens. A: Non-injected PDR group; B: Injected PDR group. *P<0.05 *vs.* control group, **P<0.01 *vs.* control group, #P<0.05 *vs.* injected PDR group, and ##P<0.01 *vs.* injected PDR group.**

Spearman's correlation analysis results

Pairwise comparison and Spearman correlation analysis were conducted for numerical results from RT-PCR in control group, injected PDR group and non-injected PDR group. It was shown that there were significantly positive correlations between the expressions of HIF-1α and VEGFR2 (*t*=0.881, P<0.05), VEGF and VEGFR2 (*t*=0.884, P<0.05), and HIF-1α and VEGFR (*t*=0.841, P<0.05) (Figure 5).

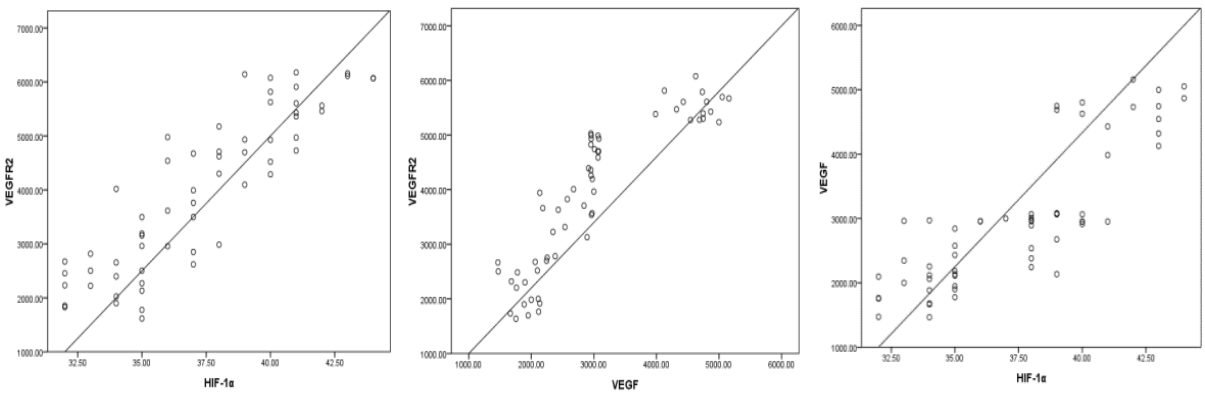

Figure 5**. Correlations among mRNA expressions of HIF-1α, VEGF and VEGFR2.**

DISCUSSION

The primary pathological changes of DR, a chronic disease of multi-factorial origin, include alteration of blood flow state in retinal capillaries, dysfunction and apoptosis of pericytes and endothelial cells and increase in vascular permeability. Such fundal changes as microangioma, petechial and patchy hemorrhage, cotton-wool spots and venous beading are defined as non-PDR. With the further aggravation of capillary occlusion, the formation of non-perfusion region triggers the neovascularization to cause repetitive hemorrhage and fibroplasia of retina, thus leading to PDR [12]. Neovascularization and fibrosis are typical pathological characteristics of PDR, which are the most important pathological factors for visual loss of patients. The pathogenesis of DR is complex and associated with multiple pathways and factors, including formation of advanced glycation end products, oxidative stress, up-regulation of matrix metalloproteinases, polyol pathway, production of growth factors and secretion of adhesion factors [13]. A long-term hyperglycemia in the body is the basis of retinal microangiopathy, and the course of DM, HbAlc level, renal function, blood lipid level, blood pressure, eating and lifestyle habits and other factors are correlated with the occurrence and development of DR [14].

HIF-1α, an oxygen-dependent transcriptional activator, is a main signaling protein regulating angiogenesis and can induce the expressions of over 70 transcription factors such as erythropoietin, glycolytic enzyme, inducible nitric oxide synthase and VEGF [15]. Moreover, VEGF can specifically bind to vascular endothelial cells and serve as a powerful angiogenic agent and a core pathogenic factor for PDR. VEGF is lowly expressed in normal retinal tissues, regulates physiological angiogenesis and exerts neuroprotective effects, but its expression is up-regulated in DR patients under the action of many pathological mechanisms.

In the present study, the expressions of HIF-1α and VEGF were raised remarkably in the fibrovascular membrane in injected PDR group and non-injected PDR group compared with those in control group, for which the possible reason is that there is neither sprouting and formation of new vessels in macular epiretinal membrane tissues nor activation of the VEGF signaling pathway in control group. Moreover, no hypoxic environment was generated in the macular epiretinal membrane tissues in control group. In normal organisms, HIF is constantly expressed and degraded to maintain its expression at a normal level [16]. In addition, injected PDR group had lower expression levels of HIF-1α and VEGF in fibrovascular membrane than non-injected PDR group. It may be because that the anti-VEGF drug injected results in atrophy and regression of some new retinal vessels, thereby ameliorating the hypoxic environment in fundus and lowering HIF-1α-related protein expression level. Furthermore, the relative mRNA expression levels of HIF-1α and VEGF in the fibrovascular membrane declined in injected PDR group in contrast with those in non-injected PDR group, and the immunohistochemical results displayed that HIF-1α and VEGF were mainly expressed in new vascular endothelial tissues, suggesting that HIF-1α and VEGF are affected by the VEGF signaling pathway and jointly regulate the neovascularization in PDR patients.

Currently, it is generally believed that the signal transduction between VEGF and VEGFR2 is the most important mediation pathway of the VEGF signaling pathway, and the expression level of VEGFR2 can indirectly reflect the degree of fundus neovascularization [20]. In this study, VEGFR2 expression was detected using RT-PCR to determine the activity of the VEGF signaling pathway. The results manifested that the expression level of VEGFR2 in pathological specimens was elevated in injected PDR group and non-injected PDR group compared with that in control group, and injected PDR group exhibited a lower mRNA expression level of VEGFR2 than non-injected PDR group. Besides, according to the immunohistochemical staining results, VEGFR2 was primarily expressed in new vascular endothelial tissues, and it was found through Spearman correlation analysis that the expressions of HIF-1α and VEGF had distinctly positive correlations with VEGFR2 expression in control group, injected PDR group and non-injected PDR group, implying that VEGFR2 expression is influenced by the VEGF signaling pathway, and VEGFR2 may modulate neovascularization in PDR together with HIF-1α and VEGF.

In conclusion, HIF-1α, VEGF and VEGFR2 are highly expressed in the fibrovascular membrane from PDR patients, and the VEGF signaling pathway may play a certain role in the formation and development of new vessels in the case of PDR. Considering the high blindness rate of PDR, exploring the functions of HIF-1α and VEGF in the pathogenesis of PDR can offer novel targets for the prevention and treatment of PDR in clinic.

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