MiRNA-21-HIF-1 α -VEGF Axis Is Associated with Myopic Choroidal Neovascularization in Guinea Pigs

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Keywords

MiR-21 · Hypoxia-inducible factor-1 α · Vascular endothelial growth factor · Myopic · Choroidal neovascularization · Guinea pig

Abstract

Introduction: Myopic choroidal neovascularization (CNV) often causes serious damage to central vision. The mechanisms behind it remain unclear. **Method:** In this study, monocular form deprivation was applied to induce high myopia, and 532-nm laser was employed to induce CNV in guinea pig. The development of neovascularization was measured comprehensively by fundus fluorescein angiography, optical coherence tomography and hematoxylin and eosin staining. Gene expression was detected by real-time polymerase chain reaction and immunohistochemistry. **Results:** The proliferation of new blood vessels increased with time and peaked at 21 days. At each time point after laser photocoagulation, the incidence of CNV was higher in form-deprived myopia (FDM) group than in control group. Myopic CNV started earlier and decreased more slowly. The obvious continuous fluorescein leakage could last as long as 1 month. The expressions of hypoxia-inducible factor (HIF)-1 α and

vascular endothelial growth factor (VEGF) increased and peaked at 14 days in both groups after laser photocoagulation. Moreover, after laser photocoagulation, miR-21 expression was upregulated in both groups, reached a peak at 7 days, with a level much higher in FDM group. In addition, miR-21 expression was positively correlated with VEGF and HIF-1 α expression in both groups. **Conclusion:** miR-21 correlated with HIF-1 α -VEGF signaling pathway may promote CNV formation in high-myopia guinea.

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Introduction

Pathological myopia (PM) often develops with choroidal neovascularization (CNV) [1]. Myopic CNV (mCNV) is characterized by the ingrowth of new and fragile blood vessels beneath retinal pigment epithelium (RPE) and/or retina in the myopic eye [2]. The outcome of myopic CNV is poor. The best corrected visual acuity of ≤ 0.10 is found in 90% of patients at 5 years after CNV [3]. Moreover, the visual acuity continues to decrease in the long

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term, mainly due to CNV-related macular atrophy. The pathogenic mechanism underlying this disease remains unclear, although several speculations have been released [4–6].

Hypoxia functions in the development of myopic CNV. Hypoxia-inducible factor (HIF)-1 participates in oxygen and energy homeostasis. Under hypoxia, HIF-1 is activated with the stabilization of its subunit HIF-1 α [7], thus stimulating the expression of VEGF, platelet-derived growth factor-B, placental growth factor, stromal-derived growth factor-1, and their receptors [8]. In a mouse model of ischemic retinopathy, HIF-1 α level increased with the expression of VEGF [9]. These findings imply that HIF-1-regulated genes may be involved in CNV, since VEGF serves as the main stimulating factor of angiogenesis [10–12]. Recently, anti-VEGF agents have been proposed as the first-line therapy for subfoveal and juxtafoveal myopic CNV [13]. However, no experimental evidence has been derived to confirm this implication.

MiRNAs regulate a wide range of pathophysiological processes [14–18]. miR-21 activates various signal pathways to enhance the expression of HIF-1 α and VEGF, resulting in angiogenesis [11]. In our previous study [19], we confirmed that miR-21 expression was positively correlated with VEGF-A and HIF-1 α levels. Subconjunctival injection of antagomir-21 could restrain corneal neovascularization by reducing the expression of HIF-1 α and VEGF-A via Sprouty 2/4-mediated inactivation of p-ERK. Expression of miR-21 also rose obviously in a mouse model of ischemic retinal neovascularization [20]. However, no report has been released about the relationships of miR-21 with VEGF and HIF-1 in myopic CNV. The present study aimed to verify these relationships using a model of choroid neovascularization based on guinea pigs with high myopia.

Materials and Methods

Form-Deprived Myopia in Guinea Pigs

Sixty 14-day-old guinea pigs were purchased from Songjiang Animal Centre (Shanghai, China) and reared under a 12-h light/12-h dark cycle (1,000 lux light level at the cage floor) in the animal facility. All guinea pigs had free access to food and water, and fresh cabbage was provided twice daily. All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Ethics Committee of Shanghai Tenth People's Hospital. Guinea pigs were randomly divided into two groups (30 guinea pigs in each group): form-deprived myopia (FDM) group and control group (without any treatment). The right eyes in FDM group were completely covered with translucent goggles

(latex gloves). For three times a day, the form-deprived eyes were checked to ensure tight coverage but no pressure on the eyes. The refractive error was measured by retinoscopy (YZ-6F, Suzhou, China) and the axis length by A-scan (KN-1800, Suzhou, China).

Induction of CNV

After 6 weeks of form deprivation, the guinea pigs were anesthetized with intraperitoneal injection of 1% sodium pentobarbital (45 mg/kg body weight). Pupils were dilated with tropicamide (5 mg/mL) and phenylephrine hydrochloride (5 mg/mL). Retinal laser photocoagulation was performed on the right eyes of the twenty-five guinea pigs selected from each group ($n = 25$). At 1–1.5 PD (papillary distance) to the papillary disc, six to eight laser spots (power: 1,500 mW; spot diameter: 50 μ m; exposure time: 0.05 s) were generated by a diode-pumped frequency-doubled, 532-nm laser (VISULAS 532s; Zeiss, Germany). Presence of a subretinal bubble indicated the rupture of Bruch membrane. After laser photocoagulation, the right eyes of FDM group were still covered to prevent the reversion of diopter.

Fundus Photography and Fundus Fluorescein Angiography

The animals were raised for 7 days, 14 days, 21 days, 28 days, and 35 days after laser treatment. Guinea pigs were anesthetized with intraperitoneal injection of 1% sodium pentobarbital (45 mg/kg body weight), and pupils were dilated with 5 mg/mL tropicamide and phenylephrine hydrochloride 30 min before examinations. One operator held the guinea pig and fully exposed its eyeballs, while the other operated the fundus camera [21, 22]. The camera was fixated, allowing fundus structures to move into the field of view. Multiple photos were taken, and the clearest picture was used for analysis.

Then, the fundus camera was turned to fundus fluorescein angiography mode, and 10% fluorescein sodium injection (0.2 mL/100 g) was injected into the peritoneal cavity of the subject. Fluorescein images were observed for 30 min. The status of CNV was evaluated with late stage (>10 min) fluorescein leakage. The laser spots were divided into four grades according to the degree of fluorescein leakage [23]: grade 1, laser spot had no high fluorescence; grade 2, laser spot had high fluorescence but no fluorescein leakage; grade 3, laser spot had high fluorescence, with mild fluorescein leakage that had not exceeded the edge of the spot; and grade 4, laser spot had high fluorescence, with significant fluorescein leakage exceeding the edge of the spot. Grade 4 spots were recorded for subsequent analysis. Quantitative evaluation of fluorescein leakage at late stage (20 min) was also performed: (1) calculating the average number of fluorescein leaks (number/eye), fluorescein leakage efficiency = (grade 4 spot/total laser points) $\times 100\%$ and (2) using image analysis software Image-pro Plus 6.0 to measure the average leakage area in late stage (by pixel area).

Optical Coherence Tomography

Anesthetized and pupils-dilated, the guinea pig was placed in a proper position by one operator to fully expose its eyeball. The other operator adjusted the optical coherence tomography (OCT) lens until clear retinal image appeared. After the optic disc was located, vertical and horizontal linear scan with a fixed length of 3 mm were performed at about 2.0 mm to the optical disc [24]. The highly reflective shuttle-shaped structure between retina and RPE layer was CNV. OCT image software was used to measure the largest central thickness in CNV [25]. The distance between the bot-

Fig. 1. Refraction and axial length in two groups before and after laser. **a** Refraction diopter. **b** Axial length. FDM versus control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

tom of choroid and vertex of CNV (T) and the surrounding normal choroid layer thickness (C) was measured (thickness of CNV = T - C). A total of 15 laser spots captured at one time point were selected in each group, and the measurement was repeated for three times, and mean value of CNV thickness was calculated.

Hematoxylin and Eosin Staining

The guinea pigs in two groups were executed through overdose anesthesia using 4% pentobarbital. Eyeballs were removed completely, trimmed, and fixed for 48 h. After conventional gradient ethanol dehydration and xylene vitrification, the tissue was embedded in paraffin wax. Continuous slices including the cornea and the optic disc were performed, and those containing the optic disc structure were selected for regular hematoxylin and eosin staining. Image-pro Plus 6.0 software was used to compare the changes of retina, choroid, and sclera at 2.0 mm to the optic nerve, as well as the thickness of CNV.

Nuclear Acid Extraction, Real-Time Polymerase Chain Reaction Analyses

Total RNA was extracted from RPE-choroid-scleral complex and quantified by measuring OD260 and OD280 (optical density 260/280 higher than 1.9). cDNA was synthesized with 5 µg of total RNA, 2 µL of random 6 mers, 0.5 µL of Oligo dT Primer, 2 µL of PrimeScript™ Buffer, and 0.5 µL of PrimeScript™ RT Enzyme Mix I (TaKaRa Bio, Otsu, Shiga, Japan) at 37°C for 15 min. The reaction was suspended at 85°C for 5 s. Conditions for PCR were 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 34 s, and 1 cycle of 95°C for 15 s and 50°C for 60 s and 95°C for 15 s. Each cDNA sample was analyzed in duplicate. Primer sequences were miR-215'-CGGCG-TTAGCTTATCAGACTGA-3' and 5'-CCAGTGCAGGGTCC-GAGGTAT-3'; VEGF 5'-CAAGATCSGCAGASGTGTAA-3' and 5'-CAASGSGAGTCTGTGTTTCT-3'; HIF-1α 5'-TGGAGATGT-TGGCACCCTA-3' and 5'-TTGAGTCTGCTGGAATGCTG-3'; GAPDH 5'-GCCCTCAATGACCACTTTGT-3' and 5'-TGCTG-TAGCSGAATCATTTG-3'.

Immunohistochemistry

The paraffin sections were baked in an oven at 100°C for 30 min, then dewaxed with xylene. After transferred to 100%, 95%, and 75% alcohol for 2 min, respectively, the sections were incubated in 3% H₂O₂ solution in methanol at room temperature for 10 min to block endogenous peroxidase activity. Having been rinsed in 300 mL of PBS for 3 times, the sections were incubated in 300 mL of citrate buffer at 100°C for 20 min and at room temperature for 20 min, then rinsed in 300 mL of PBS for 3 times. Blocking buffer (100 µL, 5% normal goat serum) was added onto the sections for 20 min. Then, the sections were incubated with 100 µL of diluted primary antibodies against rat VEGF (polyclonal, Sangon Biotech, China; diluted 1/150) and against rat HIF-1α (polyclonal, Sangon Biotech, China; diluted 1/200) overnight at 4°C. Having been washed in 300 mL of PBS for 3 times, the slides were cultured with 100 µL of diluted biotinylated secondary antibodies at room temperature for 30 min. Having been washed in 300 mL of PBS for 3 times, the slides were treated with 100 µL of DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% H₂O₂ in PBS). Having been washed in 300 mL of PBS for 3 times, the slides were counterstained in hematoxylin for 2 min. Having been rinsed in running tap water for over 15 min, the slide was dehydrated through 4 changes of alcohol (95%, 95%, 100%, and 100%), 1 min each. Stained sections were observed under microscope.

Statistical Analysis

Each experiment was repeated for at least three times. Data were shown as mean ± SD and analyzed using SPSS 18.0. Statistical comparisons between groups were performed using independent Student's *t* test, and Spearman's correlation analysis was applied to analyze the correlation between the selected indicators. A two-tailed *p* value <0.05 was considered to indicate statistical significance.

Fig. 2. Fluorescein leakage at different observation times in FDM and control group before and after laser photocoagulation.

Table 1. Effective leakage rate and mean number of level 4 laser spots at five time points

After laser	7 d	14 d	21 d	28 d	35 d
Effective leakage rate, % (n/N)					
FDM	9.1 (3/33)	23.5 (8/34)	55.5 (19/36)	51.5 (17/33)	50.0 (16/32)
Control	0 (0/33)	18.2 (6/33)	48.6 (17/35)	43.8 (14/32)	38.2 (13/34)
Mean number					
FDM	0.60±0.89	1.60±0.55	3.80±0.84	3.40±0.55	3.20±0.45
Control	0.00±0.00	1.20±0.45	3.40±1.14	2.80±0.84	2.60±0.55

Results

Changes in Diopter and Axial Length in the Guinea Pig Model

Before form deprivation, the right eyes of FDM and control group in guinea pigs were exposed to hyperopia. After 6 weeks of form deprivation, diopter ranged from -6.00 to -10.00 D (average, -7.36 ± 1.25 D), and axis length increased (average, 8.29 ± 0.71 mm). In control group, the average refraction diopter was 2.25 ± 1.50 D and the average axis length was 7.46 ± 0.57 mm. At each time point, both values were higher in the guinea pigs in FDM group, which remained far-sighted till 14 weeks. These results indicated that the model of high myopia had been successfully established through the induction of form deprivation. CNV was induced by high energy (1,500 mW) laser breaking through Bruch film. With continuous form deprivation, diopters and axial lengths kept increasing in FDM group and reached -12.84 ± 1.55 D, but the change rate decreased after laser (Fig. 1).

Changes in Fundus and CNV

Before laser photocoagulation, the retina had no fluorescein leakage in all guinea pigs; the retina and choroid were obviously thinner in FDM group than in control group; the structures of retina and choroid were relatively complete in control group. After laser photocoagulation, CNV formed in both groups, but with obvious structural difference. Fluorescein leakage appeared in FDM group at 7 days and at 14 days in control group. Fluorescein leakage rate was higher in FDM group than in control group ($p < 0.01$, respectively). The rate peaked at 21 days, then decreased in both groups, more sharply in control group (Fig. 2; Table 1). The average number of grade 4 laser spots was also larger in FDM group than in control group (Table 1). Consistent with the fluorescein leakage rate, the average leakage area peaked at 21 days, then decreased gradually in two groups. At 7 days, 14 days, 21

Fig. 3. Mean leakage area in lesions at different time points after laser photocoagulation. FDM and laser versus control and laser: * $p < 0.05$, ** $p < 0.01$; 21 days versus 35 days: # $p < 0.05$, $Np > 0.05$.

days, 28 days, and 35 days after photocoagulation, the average leakage areas at late stage of fundus fluorescein angiography were all larger in FDM group than in control group ($p < 0.05$, respectively, Fig. 3). The average leakage areas at 21 days and 35 days were statistically different in control group ($p = 0.037$), but not in FDM group ($p = 0.074$). These results indicated that retinal laser photocoagulation had successfully induced CNV in both groups. CNV was more obvious and persistent in FDM group.

On OCT, CNV was first detected as a highly reflective mass at 7 days in FDM group and at 14 days in control group, and took on its typical features at 21 days in both groups (Fig. 4a). At 7 days, 14 days, 21 days, 28 days, and 35 days after laser photocoagulation, the average CNV thickness in FDM group was 40.59 ± 5.16 , 55.80 ± 10.40 , 68.89 ± 8.56 , 66.57 ± 16.12 , and 63.59 ± 7.93 μm in FDM

(For legend see next page.)

group, and $0, 41.76 \pm 9.22, 61.53 \pm 9.45, 56.42 \pm 9.09$, and $50.56 \pm 10.20 \mu\text{m}$ in control group, the differences were statistically significant (Fig. 4b, $p < 0.05$, respectively).

At 7 days after laser photocoagulation, hematoxylin and eosin staining showed sporadic CNV in FDM group (Fig. 4a). In control group, no CNV developed, but the

outer retina was seriously damaged, with apparent infiltration of inflammatory cells and proliferation of RPE cells. CNV was observed in control group at 14 days. Both groups showed the peak of CNV at 21 days, and the decrease of inflammatory cells, increase of collagen fibers and formation of fibrous vascular tissue at 35 days. At 7

Fig. 5. VEGF and HIF-1 α expressions detected by immunohistochemistry at different time points before and after laser photocoagulation ($\times 400$).

Fig. 4. a OCT and HE sections at different time points before and after laser photocoagulation. There were CNVs in FDM group, but only outer retinal structure damage in control group at 7 days. Mean CNV thickness measured by OCT (**b**) and HE stained (**c**) section at different time points after laser photocoagulation: $*p < 0.05$, $***p < 0.001$ versus control and laser; 21 days versus 35 days: $**$ (the CNV thickness at 21 days and 35 days after laser treatment in control group) $p < 0.01$, N (the CNV thickness at 21 days and 35 days after laser treatment in FDM group) $p > 0.05$. HE, hematoxylin and eosin.

Fig. 6. Relative levels of VEGF mRNA (**a**), HIF-1α mRNA (**b**), and miR-21 (**c**) at different time points before and after laser photocoagulation.

Table 2. Integral optical density of VEGF and HIF-1α positive expression in laser spots at five time points

After laser photocoagulation	7 d	14 d	21 d	28 d	35 d
VEGF					
FDM	1,039±109	3,918±125	2,648±156	998±64	754±55
Control	591±148	2,494±133	1,853±119	644±127	336±93
<i>p</i> value	<0.001	<0.001	<0.001	<0.001	<0.001
HIF-1α					
FDM	331±61	6,319±289	3,798±432	2,366±328	1,122±157
Control	235±39	4,910±438	2,599±223	1,489±474	486±99
<i>p</i> value	<0.001	<0.001	<0.001	<0.001	<0.001

p: FDM versus control.

Fig. 7. a, b Spearman's correlations of miR-21 and VEGF mRNA level, HIF-1α mRNA level.

days, 14 days, 21 days, 28 days, and 35 days after laser photocoagulation, the average CNV thickness was 31.66 ± 3.49 , 45.34 ± 9.35 , 57.38 ± 5.46 , 56.07 ± 5.69 , and $53.66 \pm 7.00 \mu\text{m}$ in FDM group, and 0 , 33.02 ± 5.32 , 53.19 ± 4.43 , 51.65 ± 5.56 , and $47.70 \pm 6.07 \mu\text{m}$ in control group, with statistical differences (Fig. 4c, $p < 0.05$, respectively).

VEGF and HIF-1α Were Upregulated in Pathologic Myopia and Laser-Induced CNV

Before laser photocoagulation, VEGF expression was detected in RPE cells, choroid endothelial cells, the photoreceptor layer, the inner and outer nuclear layers, and the ganglion cell layer. The expression of VEGF was higher in FDM group than in control group. After laser photocoagulation, HIF-1α was expressed in FDM group, but not in con-

trol group (Fig. 5). The expressions of VEGF and HIF-1α increased in both groups, reached peak at 14 days. The range of change in the FDM group was greater than that in the control group ($p < 0.05$, Table 2). The mRNA levels of VEGF and HIF-1α expression confirmed these results ($p < 0.05$, Fig. 6a, b). No VEGF and HIF-1α expressions were detected in the sclera before and after laser photocoagulation in both groups. The results indicated that VEGF and HIF-1α upregulation were related with PM and angiogenesis, but not the extension of optic axis.

Expression of miR-21 Was Related with HIF-1α and VEGF in Myopic CNV

We assessed the relevance of miR-21 expression with the formation of myopic CNV. There was no statistical

difference in miR-21 expression between FDM group and control group ($p > 0.05$) before laser photocoagulation. After laser photocoagulation, miR-21 expression increased in both groups ($p < 0.05$) and peaked at 7 days, obviously higher in FDM group than in control group ($p < 0.05$, Fig. 6c). Moreover, the mRNA level of HIF-1 α expression rebounded transiently in FDM group at 28 days. Linear correlation analysis (Fig. 7) showed that the mRNA level of miR-21 expression was positively correlated with those of HIF-1 α and VEGF expression in FDM group ($r = 0.598, 0.663$; $p < 0.01$) and control group ($r = 0.927, 0.806$; $p < 0.001$). The miR-21 expression started to rise before the retinal structure changed, but its variation curve was parallel to the latter, suggesting that miR-21 might promote the formation of CNV through HIF-1 α -VEGF signal pathway.

Discussion

Several pathologies are implicated in retinal angiogenesis, such as macular degeneration, diabetic retinopathy, cancer, and high myopia. Myopic CNV arises from the excessive growth of ocular axis and the formation of posterior scleral staphyloma, two processes that may involve inflammatory response, angiogenesis, and protease hydrolysis. In this study, we further found that high miR-21 expression may promote CNV formation in high-myopic guinea pigs through the HIF-1 α -VEGF signaling pathway.

Few animal model studies on myopic CNV have been carried out. In this study, monocular form deprivation and 532-nm laser were used to induce myopic CNV in the guinea pig model. Form deprivation and defocus induction are the most commonly used methods to establish animal models of myopia. Guinea pigs are usually used to make models of myopia, due to their similar eyeball anatomy and emmetropization with humans [26, 27]. Laser-induced CNV mouse model is a standard model [28]. Wang et al. [29] established a guinea pig model of congenital myopic CNV induced by Krypton laser, proving its superiority to histology analysis and choroid film observation. In this model, the pathological processes of human myopic CNV can be simulated, such as the development of vascular endothelium from choroidal capillaries, the homology of RPE cells and the injury of RPE-Bruch membrane-choroidal capillary complex. However, the difference is obvious in myopic CNV between humans and guinea pigs. Human myopic CNV is mainly caused by the excessive growth of PM axis, progressive expan-

sion of posterior pole, and degeneration of retinal RPE layers, Bruch's membrane and choroid. Then, ischemia and hypoxia lead to inflammation, angiogenesis, and proteolysis in the tissue. In the animal model, however, RPE cells and Bruch membranes are selectively destroyed by mechanical, thermal, or solar energy, during which new blood vessels generate to repair the retina [30]. Therefore, more experiments are needed to confirm the reliability and practicability of this model.

In previous rat or mouse models of CNV [28, 31], the neovascularization initiated at 3–5 days after laser treatment and peaked at 7–14 days, followed by gradual fibrosis. In the present guinea pig model of high myopia, each layer in the posterior pole thinned and the blood flow in the choroid reduced due to axial growth; besides, the laser damaged the photoreceptor outer segment, RPE cells, Bruch's membranes and choroid capillaries through mechanical, thermal, and photochemical interventions. Both mechanisms lie under ischemia and hypoxia in the photocoagulated area and adjacent tissues, and angiogenesis in the retinal choroid [32]. We found that CNV appeared in guinea pigs with high myopia at 7 days after laser treatment, expanded and peaked at 21 days, till fibrovascular tissue came into shape at 35 days, which is consistent with those found in rats, mice, and primates [31, 33]. These processes are similar to those in wound healing process during which tissue integrity is restored through inflammatory reaction, angiogenesis, and fibrosis [34].

We compared the CNV induced by high myopia and CNV not induced by myopia, finding that CNV changed in the same manner in two groups. However, at each time point after laser, the rate of effective fluorescein leakage, the average area of fluorescein leakage and the average CNV thickness in FDM group were significantly higher than those in control group. In addition, we also found that myopic CNV set on earlier (7 days vs. 14 days) and degenerated more slowly in FDM group, compared with control group. We speculated that the hypoxia in the fundus was aggravated due to the combined action of myopia and laser, thus facilitating CNV development; besides, hypoxia-induced CNV fibrosis was decelerated with the continuous increase in myopia diopter and axial length. These factors also increased the incidence and prolonged the duration of high-myopia CNV at each observation time point. These findings are different from those obtained in CNV models induced only by laser [28, 31, 33].

Hypoxia induces CNV relying on the activation of some transcription factors. Previous studies have con-

firmed that tissue ischemia and hypoxia lead to the formation of CNV through HIF-1-VEGF signaling pathway [7]. As the main factor for hypoxia, HIF-1 accumulates without being degraded by proteases under hypoxia and binds to target genes carrying HRE specific regions to enhance its expression. HIF-1 is most likely to target VEGF to stimulate vascular growth [9, 12]. Previous studies have shown overexpression of VEGF and HIF-1 in surgically removed human CNV membranes [35, 36]. Yang et al. [7] also found that the mRNA and protein levels of HIF-1 and VEGF expression increased in laser-induced RAT CNV. This finding was further verified in our CNV model. After laser treatment, the mRNA and protein levels of HIF-1 and VEGF expression increased significantly, peaked at 14 days, then decreased as CNV gradually dwindled, indicating that HIF-1-VEGF is closely related to the development of severe myopic CNV.

MiR-21 serves as an angiogenesis-promoting gene in various tumors [17, 37]. Guduric-Fuchs et al. [38] found that miR-21 was highly expressed in bovine retinal microvascular endothelial cells, and miR-21 inhibitors could significantly suppress the proliferation and migration of endothelial cells, and the formation of vascular lumen. In addition, Shen et al. [20] found that miR-21 was highly expressed in the retinal tissue in a mouse model of ischemic retinal neovascularization. These studies indicate that miR-21 is closely related to ophthalmic neovascular disease. However, whether miR-21 is involved in the formation of high-myopia CNV has not been answered. We found that the expression of miR-21 was significantly up-regulated in our CNV model, suggesting its close involvement in the formation of myopic CNV. At each time point after photocoagulation, the expression of miR-21 was higher in FDM group, probably due to the combined effect of myopia and laser. Moreover, we found no significant difference in miR-21 expression between two groups untreated with laser, but mRNA and protein levels of HIF-1 and VEGF expression were still much higher in FDM group, suggesting that miR-21 is not associated with the occurrence of shade-deprived myopia. Despite the increased expression of HIF-1 and VEGF, no CNV formed in FDM group before laser, indicating that CNV involves not only high VEGF expression, but many other mechanisms, like the destruction of Bruch's membrane and RPE layers.

It has reported that miR-21 acts in response to hypoxia [39]. Myopia development is often accompanied with hypoxia [40], a key factor that induces neovascularization and may explain the high incidence of CNV in PM [41]. miR-21 is involved in the process of angiogenesis. How-

ever, the mechanisms of miR-21-mediated pathways are still unclear in mCNV. Previous studies have shown that miR-21 promotes angiogenesis by activating HIF-1 α -VEGF signaling pathways [9, 37, 42, 43]. For instance, it has been confirmed that miR-21 activates AKT/ERK1/2 signaling pathway to increase the expression of VEGF and HIF-1 α , thereby promoting neovascularization in prostate tumor [42]. In this study, we found that miR-21 level was positively correlated with HIF-1 and VEGF expression in our CNV model. After laser treatment, the peak of miR-21 expression was observed at 7 days, earlier than those of HIF-1 α and VEGF expression (14 days) and CNV formation (21 days). We speculated that miR-21 may also be involved in the formation of myopic CNV correlated the HIF-1-VEGF signaling pathway. In addition, HIF-1 α also regulates miRNA expression via their transcription factors [44]. A cross-talk between HIF-1 α and miR-21 in response to hypoxia has been reported in cardiomyocytes [45]. Regarding the correlation with HIF-VEGF, it is difficult to determine the causality between the miRNA-21 and HIF-VEGF during the mCNV formation in our study. Further research is needed to answer this question.

In conclusion, we have successfully established a guinea pig CNV model of high myopia induced by form deprivation and 532-nm laser, and teased out the relevance of miR-21 expression with the formation of myopic CNV. Our results implied that miR-21 may participate in the development of myopia CNV along with the HIF-1-VEGF signaling pathway. These findings may provide a new insights into high-myopia CNV.

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Statement of Ethics

This study protocol was reviewed and approved by the Animal Ethics Committee of Shanghai Tenth People's Hospital (SHDY-2018-3503).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.Z.: conception and design; X.M.: conduct of the study; L.L., W.D., and T.Z.: performed the experiments and analysis and interpretation of data; L.L.: wrote the paper; J.Z., X.M., and D.Z.: reviewed and edited the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated during this study are included in this article. Further enquiries can be directed to the corresponding author.

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