MicroRNA-181a or miR-29b as a diagnostic marker for neonatal retinopathy

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Introduction. To investigate the potential role of miR-181a and miR-29b in the diagnosis of retinopathy.

Methods. Serum samples from patients with retinopathy and healthy controls were measured. The association between miR-181a and miR-29b expression levels and clinical features was analyzed. In addition, the diagnostic and prognostic value of miR-181a and miR-29b in retinopathy was analyzed by ROC analysis and miR-181a and miR-29b.

Results. The expression levels of miR-181a and miR-29b were significantly lower in the serum of patients with retinopathy, and the degree of lesions in patients with retinopathy was negatively correlated with the expression levels of miR-181a and miR-29b. Moreover, miR-181a and miR-29b could distinguish retinopathy patients from normal shoushiz and with high specificity and sensitivity, while retinopathy patients with lower miR-181a and miR-29b expression showed significantly increased retinopathy compared with retinopathy patients with higher miR-181a and miR-29b expression. Correspondingly, serum VEGFA was also significantly higher in patients with retinopathy compared to healthy subjects and was negatively correlated with serum miR-181a and miR-29b expression levels.

Conclusion. miR-181a and miR-29b expression is downregulated in serum samples from patients with retinopathy and may act as diagnostic and prognostic markers of retinopathy.

Keywords. retinopathy, miR-181a and miR-29b, VEGFA, diagnosis, prognosis

INTRODUCTION

Retinopathy of prematurity (ROP) is the underdevelopment of the retina that results in disorganized growth of retinal vessels and tissues in premature infants with low birth weight associated with oxygen therapy.[1-3] The underdeveloped retinas of preterm babies are predisposed to insults that interrupt neurovascular growth, resulting in ROP. The main risk factors for the development of ROP include prematurity, low birth weight (LBW), and hyperoxia.[3,4] Retinopathy of prematurity (ROP) is a major cause of childhood blindness. Antenatal corticosteroids (ACS) exposure is known to ameliorate the risk of and mortality of neonatal morbidities.[5] An estimation of 20,000 preterm babies is blinded from ROP annually, more than half of whom were born in middle-income regions.[6] ROP, a biphasic disease, is initiated with blunted retinal vascular growth in the setting of hyperoxia (phase I), followed by abnormal retinal neovascularization in response to hypoxia-induced intraocular growth factors such as vascular endothelial growth factor (VEGF) (phase II)[7] Prematurity and long-term high oxygen supplementation have been demonstrated to be the major risk factors; whereas,maternal diseases (hypertension, diabetes mellitus and infection), caesarean section, premature rupture of membranes, respiratory distress syndrome (RDS), low Apgar score, patent ductus arteriosus (PDA), necrotising enterocolitis (NEC), intraventricular haemorrhage (IVH) and sepsis are considered as alternative risk factors[8,9]

It has been increasingly recognized that ROP differs worldwide and tailored screening and treatment approaches are needed to reduce aberrant vasoproliferation and facilitate physiologic retinal vascular development in infants.[8] Although infants with ≤1000 grams gestational weight and ≤28 weeks gestational age are more likely to have ROP, it is clear that screening for all infants at risk, regardless of gestational

weight and age, is very important in preventing ROP-related vision loss. In addition, it is also recommended to control the duration of staying in neonatal intensive care unit and oxygen therapy to as little as needed.[10] Laser photocoagulation was initially established as the standard of care by the Early Treatment for ROP (ETROP) Study.[11] However, intravitreal bevacizumab (IVB), an anti-vascular endothelial growth factor (anti-VEGF) agent, was later shown to be effective in treating ROP through the Bevacizumab Eliminates the Angiogenic Threat of ROP (BEAT-ROP) trial.[12,13] Moreover, laser photocoagulation in APROP can present technical challenges due to the presence of a persistent tunica vasculosa lentis, hazy vitreous, and difficulty in distinguishing the border between vascularized and non-vascularized retina.[13] Otherwise,the results herein indicates that infants with APROP had poor long term neurological outcomes. However, treatment with IVB and adjuvant photocoagulation, resulted in good structural and functional ophthalmic outcomes.[14]

The clinical treatment for ROP is similar to that for diabetic retinopathy (DR), retinal vascular occlusion, age-related macular degeneration, and other neovascularization diseases, which involves intravitreal injection of anti-vascular endothelial growth factor (VEGF) drugs.[15] Although this treatment is very effective, relapse and the need for repeated intraocular injections leading to retinal damage and choroidal atrophy have been shown to negatively affect the visual development of children.[16] Therefore there has been extensive research effort to discover a more suitable treatment for retinal neovascularization disease.[17] The pathogenesis of pathological RNV is multifactorial, such as angiogenesis[18], oxidative stress, and inflammation[19], and its underlying pathogenic mechanism is still not fully understood. Accumulating evidence indicates that microRNAs (miRNAs) may be aberrantly expressed and may play vital roles in the development of RNV.[20,21] MicroRNAs (miRNAs) are endogenous noncoding RNAs with a length of about 22 nucleotides which involve in the posttranscriptional regulation of gene expression[22] and regulate a wide range of physiological and pathological processes[23]. They are key regulators of vessel

development and contribute to the formation of pathological RNV.[24] Recent studies have extensively characterized the involvement of miRNA-mediated regulation in retinal angiogenesis , proliferation , apoptosis, and migration.[25]

We hypothesized that microRNAs may play a role in neonatal retinopathy. To test this possibility, we explored the expression levels of miR-181a and miR-29b in the sera of patients with retinopathy. Whether there is an association between retinal miRNAs and the development of retinal neovascularization is largely unknown. Therefore, in our study, the aim was to investigate the sera of 50 patients with retinopathy as well as 37 healthy subjects born prematurely and referred to our ophthalmology department for screening. The management of preterm infants requires an understanding of the expected difficulties and the adaptation of existing methods to their management, contributing to improve their survival and long-term prognosis.

MATERIALS AND METHODS

Sample Collection

A total of 50one retinopathy patient and37 one healthy subject's serum were used in this study. Samples were collected from2014 20187month to 5month. All study participants provided written informed consent. The study was approved by the Ethics Committee of Zibo Maternal and Child Health Hospital. All retinopathies were confirmed based on clinical presentation and imaging findings without receiving any adjuvant treatment. Samples were collected from all participants and frozen at -80°C for follow-up examination.

Peripheral blood samples were collected in serum separator tubes and processed within 2hours of collection. The blood samples were centrifuged10 at 1200g for minutes at 4°C, and the supernatant obtained was aspirated and centrifuged15 at 3000g for minutes at 4°C. Finally, the supernatant was broken up into multiple aliquots and stored at -80°C.

RT-qPCR

Real-time PCR was performed to check the expression levels of miR-181a and miR-29b. Briefly, total RNA was isolated from blood samples using TRIzol reagent (Invitrogen, Carlsbad, DE, USA). Total RNA was analyzed according to the manufacturer's instructions (ThermoScientific NanoDrop Technologies. Wilmington, DE, USA), the concentration of RNA samples was assessed based on their absorbance ratio at 260 nm / 280 nm. Afterwards, reverse transcription of cDNA was performed using a commercially available reverse transcription kit (Invitrogen). PCR was performed using the SYBR Green kit (Invitrogen) using the ABI 7500 system (Applied Biosystems, Inc., USA) according to the manufacturer's instructions. The miR-181a and miR-29b expression in each sample was normalized to U6.

Dual luciferase assay

29The miR-b and VEGFA 3'UTRs have binding sites by online software http://starase.sysu.edu.cn/index.php predicted miR-181a. PCR amplified the complementary binding sequences of miR-181a, 29miR-b and VEGFA, respectively, and VEGFA-WT and VEGFA-MUT were constructed by cloning them into pmiR-GLO luciferase vector (Promega, Madison, WI, USA). miR-181a and miR-b mimics were mixed with mimics NC and miR-181a and 29miR-b mimics, respectively. LipofectamineTM 2000 liposomes were mixed and transfected into HEK-293T cells, and luciferase activity was detected after 48 h. The experiment was repeated three times.

Data analysis methods

SPSS 21.0 (SPSS, Inc, Chicago, IL, USA) statistical software was used to analyze the data. Data were normally distributed by Kolmogorov-SmiRnov test, and results were expressed as mean ± standard deviation. t-test was used for comparison between two groups, and One-Way ANOVA one-way ANOVA and Tukey's multiple comparison test were used for comparison between multiple groups. count Fisher's exact test was used for the data; correlation analysis was performed by Pearson's correlation coefficient test; subsequently, ROC (Receiver Operating Characteristic)

curves were drawn to assess the effect of miR-181a, miR-181a and miR-b levels in serum and VEGFA levels on the results. 29P was a two-sided test and the difference was considered statistically significant at $P < 0.05$.

RESULTS

Down-regulation of miR-181a and miR-29b expression in the serum of patients with neonatal retinopathy

50 Thelevels of miR-181a and miR-29b in serum samples from patients with retinopathy and37 matched normal subjects were compared by RT-qPCR analysis. As shown in Fig1, miR-181a and miR-29b levels were significantly lower in serum from patients with retinopathy compared to normal subjects ($p < 0.001$). In addition patients with retinopathy were divided into miR-181a and miR-29b high group ($n = 23$, miR-181a and miR-29b expression levels ≤ median value9.6) and miR-181a and miR-29b low group ($n = 27$, miR-181a and miR-29b expression levels > median value9.6) based on miR-181a and miR -29b levels. As shown in the table1, we found that reduced miR-181a and miR-29b levels may be significantly associated with the degree of retinopathy ($p < 0.05$), and patients with low miR-181a and miR-29b expression had a higher degree of fibrovascular hyperplasia.

Fig 1. Downregulation of miR-181and miR-29b in neonatal retinopathy patients serum, in comparison with healthy participants. ***p<0.001.

MiR-181a and miR-29b expression in sera of patients with retinopathy may be diagnostic markers of retinopathy

ROC analysis was performed to analyze the potential diagnostic value of miR-181a and miR-29b for retinopathy. As shown in Fig 2A, the area under the curve (AUC) of miR-181a and miR-29b was 0.9312and0.9859 indicated that miR-181a and miR-29b levels were sensitive biomarkers for the diagnosis of retinopathy; in addition, miR-181a and miR-29b were also analyzed by Spearman's correlation analysis

method s correlation analysis with patients' visual acuity, we found that the expression levels of miR-181a and miR-29b in the serum of patients with retinopathy were positively correlated with patients' visual acuity, indicating that the expression levels of miR-181a and miR-29b in the serum could be used as markers for the diagnosis of retinopathy (Fig 2B).

Figure 2. miR-181a and miR-29b expression in in neonatal retinopathy patients serum may serve as a diagnostic marker for retinopathy. a. Results of receiver operating characteristic (ROC) analysis. B. Spearman's correlation analysis between serum miR-29b or miR-181a expression level and patients diopter.

miR-181a and miR-29b target VEGFA

To further clarify the mechanism of action of miR-181a and miR-29b, we used StarBase and RNA Hybird websites to predict and screen the downstream target genes of miR-181a and miR-29b, and we screened for VEGFA (Fig 3A). Subsequently, we first verified the target binding of miR-181a and miR-29b to VEGFA using dual luciferase, respectively, and we found that luciferase activity was significantly reduced in 293T cells transfected with miR-181a and miR-29b mimic, while luciferase activity in mimic NC or VEGFA-MT cells (Fig 3B~C), suggesting that miR-181a and miR-29b can have a target binding relationship with the 3'-UTR sequence of VEGFA.

Fig 3. miR-181a and miR-29b targets VEGFA mRNA 3'-UTR sequence. A. Bioinformatic analysis for predicting miR-181a or miR-29b targeted mRNA by StarBase and RNA hybrid software. by StarBase and RNA hybrid software. B~C, dual luciferase assay was utilized for confirming the bind relationship between miR-181a or miR-29b and VEGFA mRNA 3'-UTR sequence.

VEGFA is significantly highly expressed in the serum of patients with neonatal retinopathy

To determine the role played by VEGFA in neonatal retinopathy,50 we first compared the mRNA and protein levels of VEGFA in serum samples from patients

with retinopathy and37 matched normal subjects by RT-qPCR and ELISA analysis. As shown in Fig 4A~B, the mRNA and protein levels of VEGFA were significantly higher in the serum of patients with retinopathy compared with normal subjects ($p < 0.001$). Furthermore, we found that VEGFA expression levels were positively correlated with the severity of symptoms in patients with retinopathy, and patients with high VEGFA expression had a higher likelihood of fibrovascular tumor hyperplasia and a significant negative correlation with the patients' visual acuity levels (Fig 4C~D). We further analyzed the correlation between miR-181a and miR-29b expression levels and VEGFA expression levels in the serum of patients with neonatal retinopathy using Pearson's correlation, and we found a significant negative correlation between miR-181a and mIR-29b expression levels (Fig 4E~F).

Fig 4. VEGFA is upregulated in neonatal retinopathy patients serum, in comparison with healthy participants. A~B, RT-qPCR and ELISA was used to A~B, RT-qPCR and ELISA was used to determine VEGFA mRNA and protein level in serum. C, Results of receiver operating characteristic (ROC) analysis. correlation analysis between serum VEGFA expression level and patients diopter. VEGFA expression level and miR-29b or miR-181a expression level.

DISCUSSION

Retinopathy of prematurity (ROP) is a potentially sightthreatening neurovascular disease which afects the retina in infants, especially in preterm infants.[26-28] During the past two decades, the accomplishment of neonatal health care system supports a higher survival rate ofpreterm infants than ever before, leading to an increased incidence of ROP worldwide. Although there has been considerable improvement on ROP with respect to etiology, pathogenesis and treatment, it remains a leading cause of childhood blindness worldwide.[8] Since the diagnosis and treatment of ROP requires serious clinical experience, it poses certain difficulties for ophthalmologists.[29-31] Today, ROP is detected before the serious stages with the

increasing awareness of pediatricians and parents and in cases where treatment is required, intervention is made without delay.[32,33] However, risk factors such as systemic diseases, blood transfusion and sepsis couldn't be evaluated enough in our study and it makes the comparison difficult with the study. The frequency of ROP varies according to the development levels of the countries and the features of the neonatal intensive care units. In developed countries, ROP is predominantly a problem of preterms born below 28 weeks, while in developing countries it is reported that severe ROP develops up to 34 weeks.[34-36]

miRNAs play a key role in cell function and biological development, such as neurogenesis, metabolism, inflammation, and angiogenesis.[37] Recent studies have reported the dysregulation of miRNA in retinal neovascularization.[38] Further describing the role of miRNA may be potentially effective miRNA-based therapeutics for RNV. Some studies have elucidated the expression pattern of miRNAs in the OIR mouse model. We presented differentially expressed miRNAs and new miRNAs that may be involved in ischemic retinopathy (miR-3099-3p, let-7b-5p, miR-487b-3p, and miR-320-3p) and proposed miRNAs with different expressions (high or low) compared to previous studies (miR-211). In addition, among the differentially expressed miRNAs, we identified two miRNAs (miR-181a-5p and miR-21a-5p) that were significantly and robustly dysregulated as the most abundant in sequencing. Injections or other interventions to ameliorate the reduction of these microRNAs may be a new approach to prevent and treat RNV.[39] In our study, we focused on the expression levels of miR-181a and miR-29b were examined by real-time PCR, and the expression of miR-181a and miR-29b in each sample was normalized to U6. The predictive efficacy of miR-181a and miR-29b levels in serum and VEGFA levels on retinal lesions was assessed by SPSS.

MicroRNAs (miRNAs), approximately 22 nucleotides in length, induce mRNA instability or inhibit protein translation through the regulation of transcription or post-transcription gene expression.[40] The signaling of vascular endothelial growth

factor (VEGF) is one of the most potent pathways and is almost exclusively found in endothelial cells. Importantly, a large number of microRNAs (miRNAs) are responsible for angiogenesis and are expressed in endothelial cells.[41,42] Each miRNA regulates the expression of multiple protein-coding genes and therefore miRNA-based therapy provides the rationale basis for effective antiangiogenic treatment.[43] The members of the miR-181 family are evolutionarily conserved across almost all vertebrates, suggesting their functional importance.[44,45] Numerous studies have reported the involvement of miR-181a in important cell functions such as growth, proliferation, death, survival, maintenance, vascular cell signaling and blood vessel formation.[43] In our experiments, we found that reduced levels of miR-181a and miR-29b may be significantly associated with the degree of retinopathy ($p < 0.05$) and that patients with low expression of miR-181a and miR-29b have a higher degree of fibrovascular hyperplasia. We obtained downregulated miR-181a and miR-29b expression in the sera of patients with neonatal retinopathy.We also found that the expression levels of miR-181a and miR-29b in serum of patients with retinopathy were positively correlated with patients' visual acuity, indicating that the expression levels of miR-181a and miR-29b in serum can be used as markers for the diagnosis of retinopathy

To further clarify the mechanism of action of miR-181a and miR-29b, we used StarBase and RNA Hybird websites to predict and screen miR-181a and miR-29b downstream target genes, and we screened for VEGFA.In pathological conditions, there is an imbalance of proangiogenic and anti-angiogenic factors secreted by retinal endothelial cells, and the over-expression of VEGF plays an important role in the pathogenesis of ocular angiogenesis.[46] VEGFA was confirmed to be significantly highly expressed in the serum of patients with neonatal retinopathy.Finally, to determine the role played by VEGFA in neonatal retinopathy, we first compared the mRNA and protein levels of VEGFA in serum samples from 50 patients with retinopathy and 37 matched normal subjects by RT-qPCR and ELISA analysis, and

obtained a significant negative correlation between the expression levels of miR-181a and mIR-29b, that is, the lower the expression of serum miR-181a and mIR-29b, the significantly greater the degree of retinopathy.

In future studies, the genes and signaling pathways targeted by miRNAs should be further explored. Therefore, our prediction is that miR-181a and miR-29b expression is downregulated in serum samples from patients with retinopathy and may act as diagnostic and prognostic markers of retinopathy, which enables to help more clinicians to determine the extent of neonatal retinopathy and to help more patients with retinopathy to get out of harm's way sooner.

LIMITATION

Limitations of this case series include its retrospective nature and limited sample size. There were not any large infants, especially with systemic risk factors such as sepsis and blood transfusion. It is also possible to compare the risk of retinopathy in infants delivered by cesarean section compared to those delivered vaginally.

CONCLUSION

To better find diagnostic and prognostic markers for retinopathy, serum samples from subjects were analyzed and compared by RT-qPCR. miR-181a and miR-29b were added to further improve their efficiency. VEGFA was also significantly higher in the serum of patients with retinopathy and was negatively correlated with the serum expression levels of miR-181a and miR-29b. It provides a basic biological study for clinical research and acts as a diagnostic and prognostic marker for retinopathy.

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