

RESEARCH PAPER

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## Interleukin 35 regulates interleukin 17 expression and T helper 17 in patients with proliferative diabetic retinopathy

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### ABSTRACT

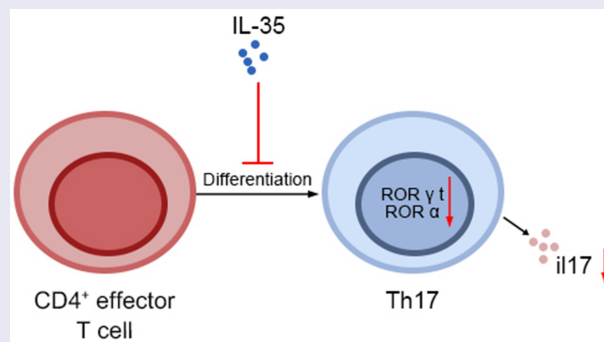
T helper 17 (Th17) cells regulate inflammatory processes and are implicated in pathogenesis of proliferative diabetic retinopathy (PDR) through modulation of interleukin-17 (IL-17). IL-35, anti-inflammatory factor, negatively mediates IL-17 expression and Th17 differentiation. In this study, the role of IL-35 in PDR was assessed. The results showed that IL-35 was down-regulated, while IL-17 was up-regulated, in peripheral blood mononuclear cells (PBMCs) of PDR patients. In addition, immunofluorescence analysis indicated that frequency of Th17 cells was enhanced in the PBMCs of PDR patients. However, incubation with IL-35 reduced the Th17 cell frequency and decreased the level of IL-17 in CD4<sup>+</sup> T lymphocytes. Moreover, the levels of transcription factors essential for Th17 differentiation, ROR  $\alpha$  (retinoid-related orphan receptor alpha) and ROR  $\gamma$ t, were reduced by IL-35 treatment. In conclusion, IL-35 reduced level of IL-17 and inhibited Th17 differentiation to protect against PDR.

### ARTICLE HISTORY

Received 7 April 2022  
Revised 10 May 2022  
Accepted 17 May 2022

### KEYWORDS

IL-35; IL-17; Th17 cell; ROR  $\alpha$ ; ROR  $\gamma$ t; proliferative diabetic retinopathy





### Highlights

- IL-35 reduced Th17 frequency and decreased IL-17 in CD4<sup>+</sup> T lymphocytes.
- IL-35 reduced levels of ROR  $\alpha$  and ROR  $\gamma$ t to inhibit Th17 differentiation.
- IL-35 protected against proliferative diabetic retinopathy.

### Introduction

Proliferative diabetic retinopathy (PDR) is a severe stage of DR, characterized by fibrovascular changes that lead to vitreous hemorrhage or traction retinal

detachment [1]. Panretinal photocoagulation and anti-vascular endothelial growth factor agents have been widely used in the treatment of PDR [2]. However, intraoperative and postoperative complications from vitrectomy surgery might constrain the benefits of surgical treatment [2]. Therefore, other strategies are urgently needed for the treatment of PDR. Increasing evidence has shown that inflammatory factors were elevated in serum of patients with PDR, local inflammation was implicated in the pathogenesis of PDR [3]. Therefore, regulation of the inflammatory factors might be promising strategies for prevention of PDR.

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T helper 17 (Th17) cells belong to CD4<sup>+</sup> T lineage, and regulate secretion of IL-17A and the development of autoimmune or inflammatory diseases [4]. IL-17 secreted by Th17 cells initiates secretion of pro-inflammatory factors and further amplifies the inflammatory response in inflammatory and autoimmune diseases [5]. Therefore, Th17 and IL-17 might be involved in pathogenesis of DR. For example, in type 2 diabetes, circulating Th17 cells were up-regulated, promoted production of IL-17 and contributed to the chronic inflammation [6]. Similarly, Th17 cell frequency and concentrations of IL-17A were enhanced in PBMCs of patients with DR [7]. Suppression of Th17 cell differentiation and down-regulation of IL-17 expression ameliorated retinal capillary degeneration to attenuate streptozotocin-induced DR [8]. Th17 cells and IL-17A were also up-regulated in vitreous fluid [9,10] or PBMCs [11] of patients with PDR. However, the effect of Th17-IL-17A pathway on PDR has not been reported yet.

IL-35, secreted by Treg cells, is an immunosuppressive factor in distinct inflammatory responses [12]. IL-35 exerted anti-inflammatory effect to alleviate streptozotocin-induced diabetic neuropathic pain [13]. IL-35 were reduced in PDR patients [14]. Moreover, IL-35 has been shown to inhibited the level of IL-17, and decrease infiltrations of CD4<sup>+</sup> T cells to prevent inflammatory diseases [15]. IL-35 also inhibited Th17 cell differentiation and down-regulated IL-17 protein expression in PBMCs of patients with periodontitis through down-regulation of regulators for differentiation of Th17 cells, ROR  $\gamma$ t and ROR  $\alpha$  [16]. Therefore, IL-35 might regulate PDR through Th17-IL-17A pathway. Effects of IL-35 on Th17 cells differentiation and IL-17 protein expression in patients with PDR were evaluated in this study.

## Materials and methods

### Sample collection

Patients with PDR (N = 21) and diabetic patients with idiopathic macular epiretinal membranes (N = 29) were recruited at the First People's Hospital of Zunyi. This study was approved by the Ethics Committee of the First People's

**Table 1.** Clinical characteristics of diabetic patients.

Characteristic	Control	PDR	P value
Number of patients	29	21	
Gender (Male/Female)	15/14	11/10	0.822
Age(years)	56.03 ± 7.32	58.05 ± 7.27	0.330
Duration of diabetes (years)	-	12.29 ± 3.89	
BMI (kg/m <sup>2</sup> )	20.42 ± 6.4	24.72 ± 3.49	0.01
FPG (mmol/L)	5.28 ± 0.51	8.47 ± 0.76	<0.001
HbA1c (%)	4.88 ± 0.56	9.2 ± 1.59	<0.001

Hospital of Zunyi (Approval No. 2021-1-003) and in accordance with World Medical Association Declaration of Helsinki, and the Ethical principles for medical research involving human subjects. Participants signed informed consents. Demographics of participants are shown in Table 1. Blood samples were acquired from the participants to collect the PBMCs according to previous study (Levels of Interleukin 27 and Interleukin 35 in the Serum and Vitreous of Patients with Proliferative Diabetic Retinopathy).

### Cell culture

PBMCs were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a 37°C incubator with 5% CO<sub>2</sub> according to a previous study (Protective effects of SIRT1 in patients with proliferative diabetic retinopathy via the inhibition of IL-17 expression).

### Enzyme linked immunosorbent assay (ELISA)

The blood samples isolated from the participants were centrifuged at 2,000 g to collect supernatants. Serum level of IL-35 was detected by ELISA kit (R&D Systems, Minneapolis, MN, USA). The isolated PBMCs were incubated with ionomycin (250 ng/mL) (Sigma-Aldrich, St Louis, MO, USA) and phytohemagglutinin (5 µg/mL; Sigma-Aldrich) for 2 days. IL-17A was also detected by ELISA kit (R&D Systems) according to previous study (Th17 cell frequency and IL-17A concentrations in peripheral blood mononuclear cells and vitreous fluid from patients with diabetic retinopathy).

### Isolation and treatment of CD4<sup>+</sup>T lymphocytes

The isolated PBMCs were incubated with CD4 Biotin-Antibody cocktail from CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and treated with Anti-Biotin micro beads. MACS separator was then used to collect CD4<sup>+</sup> T lymphocytes. The lymphocytes were incubated with 1 µg/mL anti-CD3 Mabs (Abcam, Cambridge, MA, USA), 10 µg/mL anti-CD28 Mabs (Abcam), 20 ng/mL TGF-β (Sigma-Aldrich), and 50 ng/mL IL-6 (Sigma-Aldrich) for the differentiation to Th17 cells. Differentiated Th17 cells were incubated with IL-35 (50 ng/mL; Sigma-Aldrich) for 3 days according to previous study (Th17 cell frequency and IL-17A concentrations in peripheral blood mononuclear cells and vitreous fluid from patients with diabetic retinopathy).

### Flow cytometry

The isolated PBMCs, CD4<sup>+</sup> T lymphocytes or differentiated Th17 cells were incubated with 25 ng/mL phorbol myristate acetate, Brefeldin A (1 µg/mL) and Ionomycin (1 µg/mL) (Sigma-Aldrich) for 4 hours. Cells were then incubated with fluorescein-isothiocyanate-labeled anti-CD4 (BioLegend, San Diego, CA, USA) antibody, stained with phycoerythrin-Cy7-labeled anti-IL-17 (Abcam). Th17, CD4<sup>+</sup> IL-17<sup>+</sup>, were analyzed by FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) according to previous study (Th17 cell frequency and IL-17A concentrations in peripheral blood mononuclear cells and vitreous fluid from patients with diabetic retinopathy).

### qRT-PCR (Quantitative Reverse Transcription PCR)

Isolated PBMCs, CD4<sup>+</sup> T lymphocytes or differentiated Th17 cells were lysed in TRIzol kit (Invitrogen), and the isolated RNAs were synthesized into cDNAs. PreTaq II kit (Takara, Dalian, Liaoning, China) was used to determine mRNA expression of IL-35, ROR γt, ROR α with following primers: IL-35 (F: 5'-CGGTGCCCTACATGCTAAAT-3' and R: 5'-GCGGAGTCGGTACTTGAGAG-3'); ROR γt (F:

5'-CTCCATCTTTGACTTCTCCCACTCCCTA-3' and R: 5'-CACATGCTGGCTACACAGGCTC-3'); ROR α (F: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and R: 5'-CGCTTCACGAATTTGCGTGTTCAT-3'). The relative expression of IL-35, ROR γt, and ROR α were calculated using 2<sup>-ΔΔCq</sup> method through normalization to GAPDH according to previous study (Lower level of IL-35 and its reduced inhibition in Th17 cells in patients with bone marrow mononuclear cell Coombs test-positive hemocytopenia).

### Western blot

CD4<sup>+</sup> T lymphocytes or differentiated Th17 cells were lysed in RIPA buffer (Beyotime, Beijing, China). Protein samples were then separated by 10% SDS-PAGE. Following transferred onto nitrocellulose membranes and blocked in 5% bovine serum albumin, the membranes were probed with specific antibodies: anti-ROR γt (1:2000), anti-ROR α (1:2500), and anti-GAPDH (1:3000). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:4000). Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich) according to previous study (Protective effects of SIRT1 in patients with proliferative diabetic retinopathy via the inhibition of IL-17 expression).

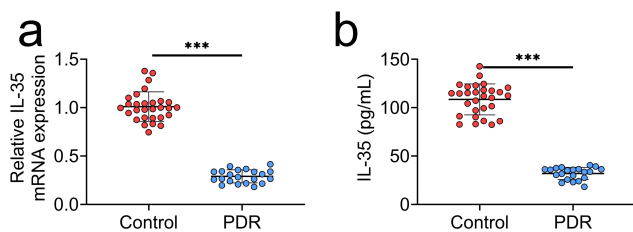
### Statistical analysis

All the data with at least triple replicates were expressed as mean ± SEM, and analyzed by Student's *t* test or one-way analysis of variance under SPSS software. A *p* value of <0.05 was considered statistically significant.

## Results

### IL-35 was downregulated in PBMCs of patients with PDR

To investigate the role of IL-35 in PDR, PBMCs were isolated from PDR patients. The mRNA expression of IL-35 was decreased in PBMCs of PDR patients (Figure 1(a)). Moreover, patients with PDR also demonstrated lower IL-35 than in



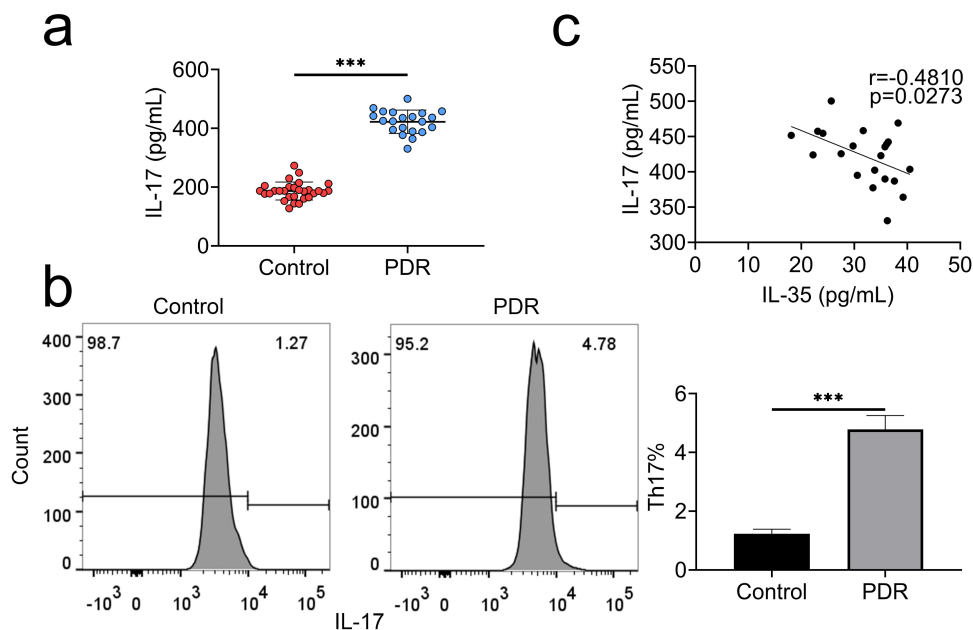
**Figure 1.** IL-35 was down-regulated in PBMCs of patients with PDR.

(a) mRNA expression of IL-35 was decreased in the PBMCs of patients with PDR compared to the control (patients with idiopathic macular epiretinal membranes). (b) Serum level of IL-35 was decreased in the PBMCs of patients with PDR compared to the control. \*\*\*  $p < 0.001$ .

patients with idiopathic macular epiretinal membranes (Figure 1(b)), suggesting the possible relation between IL-35 and PDR.

### Th17 frequency and IL-17 were upregulated in PDR

Th17-IL17 were then assessed in patients with PDR. Level of IL-17 (Figure 2(a)) and frequency of Th17 cells (Figure 2(b)) were enhanced in PBMCs of PDR patients compared to patients with idiopathic macular epiretinal membranes.



**Figure 2.** Th17 frequency and IL-17 were up-regulated in PDR.

(a) IL-17 was increased in PBMCs of patients with PDR compared to the control. (b) The frequency of Th17 cells was enhanced in the PBMCs of patients with PDR compared to the control. (c) IL-17 showed negative correlation with IL-35 expression in patients with PDR. \*\*\*  $p < 0.001$ .

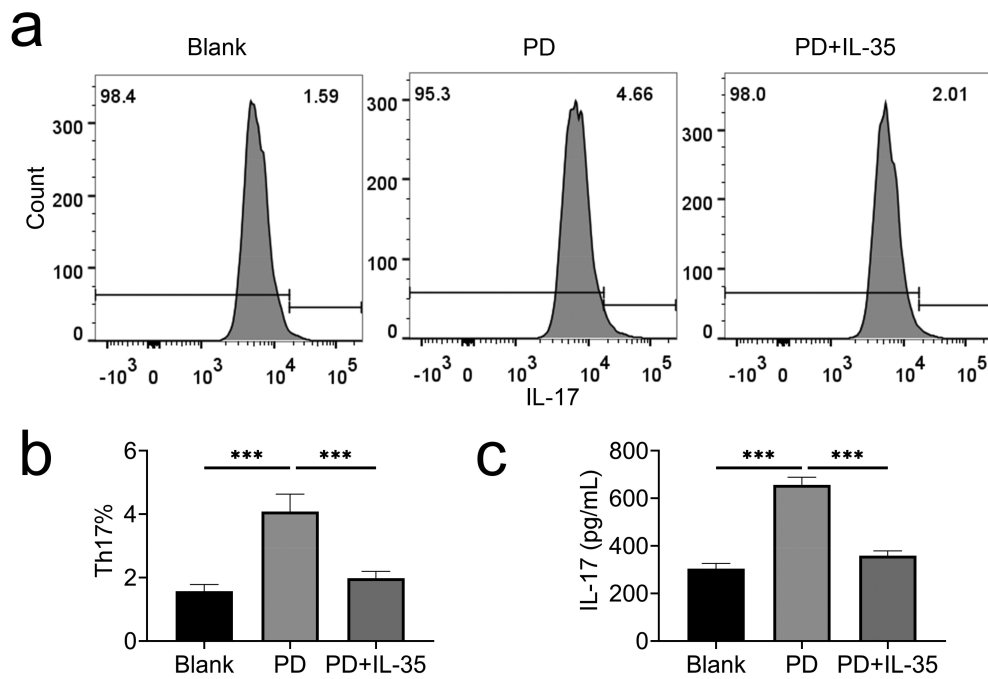
IL-17 expression showed negative correlation with IL-35 in patients with PDR (Figure 2(c)).

### IL-35 suppressed Th17 differentiation

CD4<sup>+</sup> T lymphocytes of PBMCs were incubated with IL-35 to investigate the role of IL-35 in Th17 differentiation. Th17 cell frequency was increased in CD4<sup>+</sup> T lymphocytes post differentiation (Figure 3(a)), while incubation with IL-35 reduced the frequency (Figure 3(a,b)). Moreover, IL-35 also reduced the level of IL-17 in differentiated Th17 cells (Figure 3(c)), indicating that IL-35 suppressed Th17 differentiation in PDR.

### IL-35 reduced expression of ROR $\gamma$ t and ROR $\alpha$

Underlying mechanism of IL-35-mediated Th17 differentiation was then evaluated. The mRNA (Figure 4(a)) and protein (Figure 4(b)) expression of ROR  $\gamma$ t and ROR  $\alpha$  were enhanced in differentiated Th17 cells. However, incubation with IL-35 reduced ROR  $\gamma$ t and ROR  $\alpha$  (Figure 4(a,b)), revealing that IL-35 suppressed Th17 cell differentiation through decreasing of ROR  $\gamma$ t and ROR  $\alpha$ .



**Figure 3.** IL-35 suppressed Th17 differentiation.

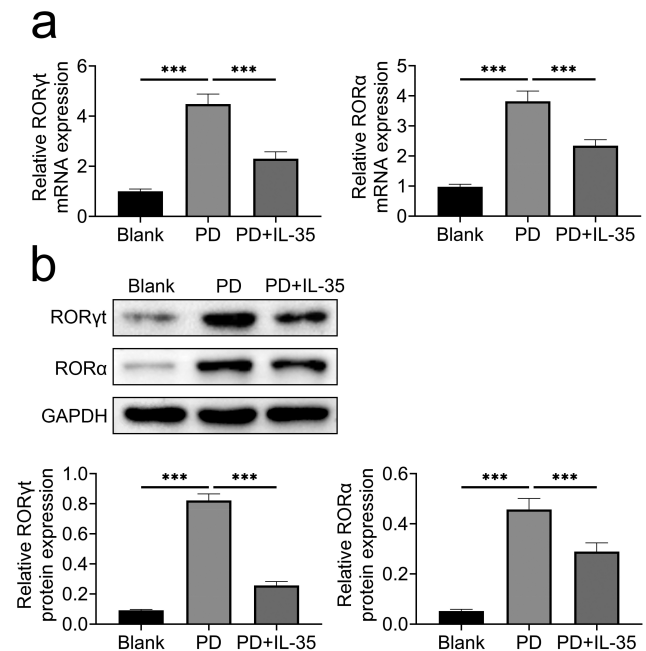
(a) Incubation with IL-35 reduced the frequency of Th17. (b) Relative frequency of Th17 cells. (c) Incubation with IL-35 reduced IL-17 in differentiated Th17. \*\*\*  $p < 0.001$ .

## Discussion

Emerging evidence has shown that inflammation functions as a key player in DR, especially PDR [17]. Th2 and Th17 cells, and the associated cytokines were enhanced in vitreous fluid of patients with PDR [10]. Inhibition of Th17 cell frequency and the related cytokine, IL-17A, attenuated retinal capillary degeneration in streptozotocin-induced diabetic mice [8]. This study for the first time showed that IL-35 repressed differentiation of Th17 cells and the production of IL-17, providing a potential strategy for the prevention of PDR.

IL-35 was highly upregulated in PDR patients [14]. IL-17A was also elevated in vitreous [3] and aqueous humor [18] of patients with PDR. This study also confirmed that IL-35 was upregulated in PDR patients, and PBMCs isolated from patients with PDR expressed higher IL-35 and IL-17 than the patients with idiopathic macular epiretinal membranes. Similarly, in line with previous studies that Th17 cells were elevated in patients with PDR [9–11], this study also confirmed the enhanced frequency of Th17 cells in patients with PDR. Moreover, IL-17 expression showed a negative correlation with IL-35 in patients with

PDR. The role of IL-35/IL-17 in PDR was then evaluated.



**Figure 4.** IL-35 reduced ROR  $\gamma$ t and ROR  $\alpha$  expression.

(a) Incubation with IL-35 reduced ROR  $\gamma$ t and ROR  $\alpha$  mRNAs in differentiated Th17 cells. (b) Incubation with IL-35 reduced ROR  $\gamma$ t and ROR  $\alpha$  proteins in differentiated Th17 cells. \*\*\*  $p < 0.001$ .

Previous study has shown that treatment with IL-35 reduced IL-17 expression and the production of Th17 cells in CD4<sup>+</sup> T lymphocytes [19]. IL-35 exerted anti-inflammatory effect against psoriasis and inflammatory bowel disease through promoting of Treg infiltration [15]. Results indicated that incubation with IL-35 recombinant protein reduced frequency of Th17 cells, and down-regulated IL-17 in the differentiated Th17 cells. Therefore, IL-35 suppressed Th17 differentiation and reduced IL-17 production, thus contributing to attenuation of PDR. Moreover, angiogenesis, with the degradation of basement membrane and formation of capillary tubes, also contribute to inflammatory responses, and promote the development of PDR [17]. IL-35 has been shown to regulate pro-angiogenic molecules through Th17/IL-17 signaling [20]. The role of IL-35/Th17/IL-17 in PDR-associated angiogenesis should be investigated in further research.

ROR  $\gamma$ t and ROR  $\alpha$  are critical regulators for Th17 differentiation, and are regarded as potential targets for Th17 cells-mediated diseases [21]. Deficiency of ROR  $\gamma$ t and ROR  $\alpha$  resulted in abrogation of Th17 differentiation [22]. IL-35 has been reported to down-regulate the expression of ROR  $\gamma$ t and ROR  $\alpha$ , and repressed frequency of Th 17 cells [16]. Here, IL-35 also attenuated Th17 differentiation-induced increase in ROR  $\gamma$ t and ROR  $\alpha$ , thus suppressing differentiation of Th17 in PDR. Therefore, IL-35 suppressed differentiation of Th17 through down-regulation of ROR  $\gamma$ t and ROR  $\alpha$  to ameliorate PDR.

## Conclusion

In summary, IL-35 functioned as an anti-inflammatory cytokine in PDR through inhibition of Th17 differentiation and down-regulation of IL-17. This study suggested the potential clinical significance of IL-35 therapy in the treatment of PDR patients, and confirmed the correlation between IL-35 and Th17/IL-17 in PDR patients. However, the role of IL-35 in *in vivo* animal model of PDR remains elusive and will be investigated in the future.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This work was supported by Zunyi Science and Technology Bureau Joint Research and Development Fund Project (Grant No. (2017)35), National Nature Science Foundation of China (Grant No. 82160200), R&D Program of The First People's Hospital of Zunyi (Grant No. (2020)4), Science and Technology Fund Project of Guizhou Provincial Health Commission in 2020 (Grant No. gzwjk2020-1-155).

## Ethics approval

Ethical approval was obtained from the Ethics Committee of the First People's Hospital of Zunyi (Approval No. 2021-1-003).

## Contribution of authors

Ai Yan and Ying Zhang designed the study and carried them out, Xiacong Wang supervised the data collection, analyzed the data, interpreted the data, Yueling Cui and Wei Tan prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

## Statement of human and animal rights

All procedures in this study were conducted in accordance with the Ethics Committee of the First People's Hospital of Zunyi approved protocols.

## Statement of informed consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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