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Original Article

MicroRNA-503 attenuates hypoxia-induced pulmonary artery smooth muscle cell proliferation through directly targeting Bcl-2

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Abstract: Emerging evidence has identified the vital role of microRNAs (miRNAs) in the etiology of pulmonary arterial hypertension (PAH). However, little is well-understood about the role of miR-503 in PAH. Herein, we aimed to determine the expression profile of miR-503 and explore its modulatory mechanism in PAH. Serum samples were collected from patients with idiopathic pulmonary arterial hypertension (IPAH) (n=31) and healthy participants (n=30), and qRT-PCR was performed to study the expression levels of miR-503. Cell proliferation and migration were assessed by MTT assay and wound healing assay, respectively. Cell apoptosis and cell cycle distribution were analyzed by flow cytometry. We found that the expression levels of miR-503 in IPAH patients' sera were evidently decreased. After transfection with miR-503 mimics, overexpression of miR-503 significantly inhibited the hypoxia-induced excessive proliferation, migration and cell cycle progression of pulmonary arterial smooth muscle cells (hPASMCs). Further, Bcl-2 was identified as a direct target of miR-503 in hPASMCs through bioinformatics and luciferase reporter analysis. Taken together, our findings provide the first clues that miR-503 serves a critical role in the pathogenesis of PAH and may serve as a potential therapeutic strategy for this disease.

Keywords: Pulmonary arterial hypertension, hPASMC, miR-503, Bcl-2, cell cycle

Introduction

Pulmonary arterial hypertension (PAH), a severe pathophysiological and hemodynamic condition of the pulmonary circulation, is defined by a mean pulmonary artery pressure (mPAP) ≥25 mmHg based on hemodynamic criteria [1]. The disease is characterized by excessive pulmonary vascular remodeling, small vessel occlusion and loss, and increased pulmonary vascular resistance which could eventually lead to right heart failure and death [2, 3]. Recent registries have shown that the prevalence of PAH in USA varies from 4.5 to 12.3 per 100,000 people [1]. Although enormous efforts have been made, PAH still confers an unacceptable high mortality rate of 10-15% per annum with a median survival of 6-7 years [4]. Accordingly, it is of great clinical significance to identify specific molecular targets and develop more effective therapies for this fatal disease.

MicroRNAs (miRNAs) are an emerging class of short non-coding RNAs (approximately 22 nucleotides long) originally found in 1993 by Lee et al. in C. elegans [5]. These RNA molecules function as negative regulators of gene expression through either degrading or repressing translation of messenger RNA (mRNA) targets [6]. Accordingly, miRNAs play pivotal regulatory roles in a variety of physiological and pathological events. Deregulation of miRNAs is associated with multiple cardiovascular diseases, including PAH. For example, downregulation of miR-126 contributes to the failing right ventricle in experimental PAH [7]. Deng et al. showed that miR-143-3p is upregulated in animal models of PAH as well as in samples from PAH patients [8]. MiR-503 has been previously reported to be closely associated with deregulated proliferation and migration of vascular smooth muscle cells (VSMCs), which play a critical role in cardiovascular disease development

Table 1. Demographic and clinical characteristics of IPAH patients and healthy controls

Characteristics	IPAH patients (n=31)	Healthy controls (n=30)	*P* value
Age (years)			0.906
≥35	14	14	
<35	17	16	
Gender			0.530
Male	12	14	
Female	19	16	
BMI (kg/m^2)			0.488
≥25	13	10	
<25	18	20	
Smoking			0.613
Smoker	9	7	
Non-smoker	22	23	
WHO functional class			
Class I, II	16		
Class III	13		
Class IV	2		
6-min walk distance (m)	411 (330-585)		
mRAP (mmHg)	8 (4-12)		
mPAP (mmHg)	61 (42-75)		
PVR (Wood Unit)	17 (8-24)		
PAWP (mmHg)	8 (5-13)		
CO (L/min)	2.9±1.1		

Values are presented as mean ± SD or median (interquartile range). BMI: body mass index; WHO: World Health Organization; mRAP: mean right atrial pressure; mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; PAWP: pulmonary artery wedge pressure; CO: cardiac output.

[9]. However, up to now, there are limited reports about the expression pattern and biological function of miR-503 in PAH.

In the present study, we took advantage of an *in vitro* model of PAH to investigate whether miR-503 attenuates the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) under hypoxia and to further explore the molecular mechanisms regulating this process. Our findings revealed that miR-503 might be a potential target in PAH therapy.

Materials and methods

Clinical samples

The present study enrolled 31 therapy-naïve patients who were diagnosed with idiopathic pulmonary arterial hypertension (IPAH) by right heart catheterization (RHC) at Chengfei Hospital (Chengdu, China) according to the updated WHO clinical classification [10], inclusion criteria including mean pulmonary artery pressure (mPAP) ≥25 mmHg, pulmonary artery wedge pressure (PAWP) ≤15 mmHg. 30 healthy participants without PAH or significant cardiorespiratory disease were also recruited. The characteristics of IPAH patients and healthy participants are listed in **Table 1**. This study was approved by the Ethics Committee of Chengfei Hospital and the written informed consents were obtained from all subjects prior to participation.

Peripheral blood samples for miRNA detection were centrifuged at 3000 g for 15 min at 4°C. Aliquots of the supernatant were transferred to RNase-free tubes and stored at -80°C until use for RNA extraction.

Cell culture, transfection and hypoxia treatment

Human pulmonary arterial smooth muscle cells (hPASMCs), purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were cultured in SmGM-2 smooth muscle cell growth medium (Lonza Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). Cells were used for experiments between passages 5 and 8.

MiR-503 mimics and corresponding negative control miR (miR-NC) were purchased from RiboBio (Guangzhou, China). Transient transfection of cells was achieved with Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Cell culture media was changed after 6 hours to remove the transfection reagent. 48 hours after transfection, transfection efficacy was verified by qRT-PCR.

For hypoxia experiments, hPASMCs were placed in a humidified airtight incubator that was constantly infused with a hypoxic gas mixture (3% O_2, 5% CO_2, and 92% N_2). The oxygen concentration was monitored continuously (Forma 3130; Thermo Scientific, Rockford, IL, USA). At the same time, normoxic hPASMCs were placed in an incubator infused with air (21% O_2, 5% CO_2 and 74% N_2).

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from serum samples and cells using mirVana™ PARIS miRNA isolation kit (Ambion, Foster City, CA, USA) according to manufacturer's instructions. The quantity and quality of the total RNA extracted were determined spectrophotometrically (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China) and RNA integrity was determined by gel electrophoresis.

Each miRNA was specifically reverse transcribed to cDNA using a TaqMan MicroRNA Reverse Transcription Kit, and the relative levels of miR-503 to the control U6 were detected by qRT-PCR using the All-in one TM miRNA qRT-PCR reagent kit (GeneCopoeia, Rockville, USA). The primers were as follows: miR-503, RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCAG-3', forward primer: 5'-TAGCAGCGGGAACAGTT-3' and reverse primer: 5'-GTGCAGGGTCCGAGGT-3'; U6, RT: 5'-AACGCTTCACGAATTTGCGT-3', forward primer: 5'-CTCGCTTCGGCAGCACA-3' and reverse primer: 5'-AACGCTTCACGAATTTGCGT-3'. The data were analyzed using $2^{-\Delta\Delta Ct}$ method. All reactions were performed in triplicate on a 7500 Fast Real-Time PCR system (Applied Biosystems, USA).

Western blot

Proteins were isolated from cells using RIPA lysis buffer (Beyotime, Beijing, China), and the protein concentration was measured by a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal quantities of proteins were loaded and separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membrane was then plotted with primary antibodies against p27 (1:1000; Cell Signaling Technology, Boston, MA, USA), Cyclin D1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (1:500; Abcam, Cambridge, UK) and Bcl-2 (1:500; Abcam) overnight at 4°C on a shaker. Then the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, and visualized using enhanced chemiluminescence assay (ECL, Thermo, Rockford, USA). β-actin was used as a internal control.

Luciferase activity assay

For the binding of miR-503 to Bcl-2 3'UTR, the 3'UTR segment of the Bcl-2 gene was amplified by PCR and inserted into the pGL3-luciferase reporter plasmid (Promega, Madison, WI, USA). A mutant construct in miR-503 binding sites of Bcl-2 3'UTR region also was generated using Quick Change Site-Directed Mutagenesis Kit (Agilent, Roseville City, CA, USA). Co-transfections of luciferase reporter plasmid and pRL-TK vector expressing the Renilla luciferase (Promega) into the miR-503 overexpressing or control hPASMCs were accomplished by using Lipofectamine 2000. Luciferase activity was measured 48 hours after transfection by the Dual-Luciferase Reporter Assay System (Promega).

MTT assay

Cell proliferation was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. hPASMCs were seeded in 96-well plates (5000 cells/well) and transfected with either miR-NC or miR-503 before exposure to hypoxia. After incubation for 48 h, 10 μl of MTT solution (5 mg/ml) was added to each well for 4 h. The 570 nm absorbance was investigated using a microplate reader (Bio-Rad, Hercules, CA, USA).

Wound healing assay

Cell migration was determined by wound healing assay. hPASMCs were seeded into 6-well plates (5×10^5 cells/well) and maintained for 24 h to reach to 90% confluency. After formation of the cell monolayer, the cells were serum-starved. Following starvation, a straight scratch was created onto the monolayer with a sterile micropipette tip. After scratching, the debris and floating cells were removed by PBS washing. Images of cells migrating into the wound were captured at 0 and 24 h after scratching using an Olympus IX51 microscope.

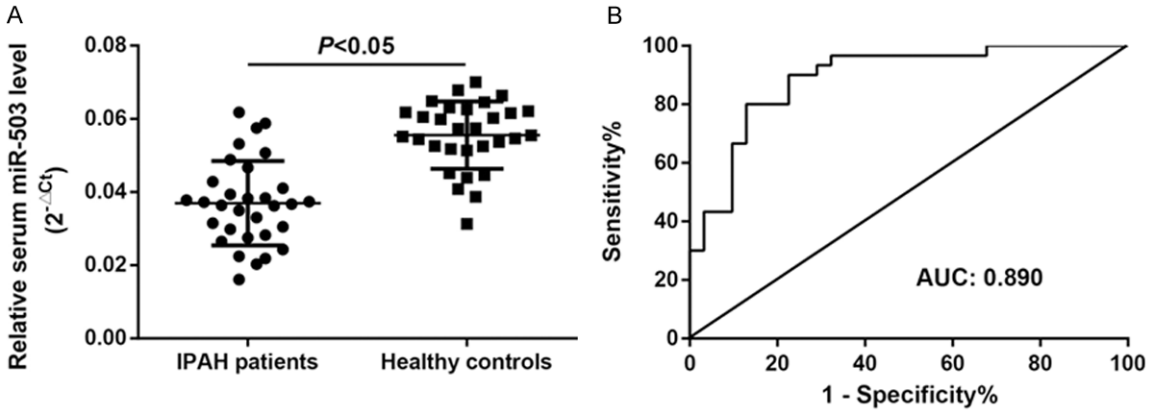

Figure 1. Serum miR-503 level is markedly reduced in IPAH patients. A. Levels of serum miR-503 in healthy controls and IPAH patients were measured. The results were expressed as $2^{-\Delta Ct}$. $\Delta Ct = Ct_{miR\text{-}503}\text{-}Ct_{U6}$. The data are expressed as mean ± SD. B. ROC curve analysis of serum miR-503 for discriminative ability between IPAH patients and healthy controls, with an AUC of 0.890.

Flow cytometry analysis

For cell cycle assay, hPASMCs were fixed in 70% ethanol at 4°C overnight. Then, the cells were analyzed by using a Cycletest™ Plus DNA Reagent Kit (BD Biosciences, Bedford, MA, USA). The cell cycle was determined with a FACS Calibur flow cytometer (BD Biosciences) and data were analyzed with ModFit 3.0 software (BD Biosciences).

The apoptosis rate of hPASMCs was tested using an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) in a FACS Calibur flow cytometer, and the data were analyzed using the Cell Quest Pro software (BD Bioscience).

Statistical analysis

Statistical analyses were conducted with GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). All *in vitro* experiments were carried out at least 3 times and results were presented as means ± standard deviation (SD). A receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of serum miR-503 for discriminating between IPAH patients and healthy individuals. Differences between groups were determined using Student's *t*-test, Chi-square test or one-way analysis of variance (ANOVA) test followed by post-hoc test when appropriate. All the differences were regarded as statistically significant when $P<0.05$.

Results

Serum miR-503 level is markedly reduced in IPAH patients

To study the role of miR-503 in PAH, we examined the miR-503 levels in sera from 31 IPAH patients and 30 healthy participants. As indicated in **Figure 1A**, the serum level of miR-503 was significantly decreased in IPAH patients than controls ($P<0.05$). Then, we examined the diagnostic efficiency of serum miR-503 in IPAH through ROC curve analysis. As shown in **Figure 1B**, the area under the curve (AUC) of the ROC curve was 0.890 (95% CI: 0.808-0.973). When the level of serum miR-503 was at the optimum cutoff point (0.0434), sensitivity and specificity were 90.0% and 77.4%, respectively. These results indicated that serum miR-503 level possessed a strong diagnostic efficiency for discriminating IPAH patients from healthy individuals.

MiR-503 overexpression attenuates hPASMC proliferation and migration induced by hypoxia

Hypoxia is a critical factor in the pathogenesis of PAH. As shown in **Figure 2A**, miR-503 expression was gradually decreased in hPASMCs after exposure to hypoxia in a time-dependent manner. To further study the biological functions of miR-503, we induced overexpression of miR-503 by miR-503 mimics in hPASMCs, and the efficiencies were confirmed by qRT-PCR. The

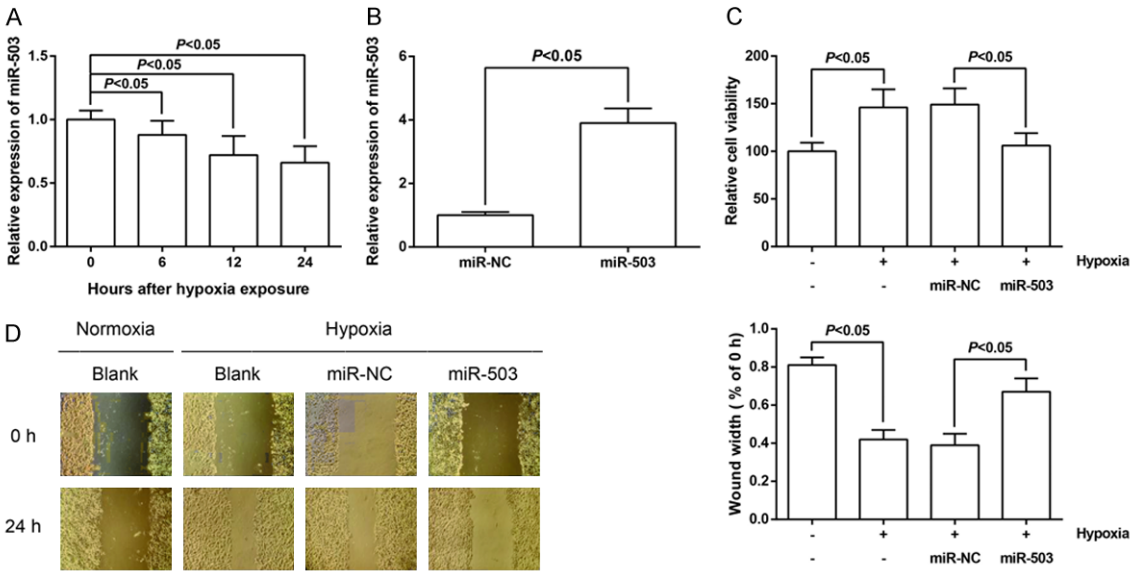

Figure 2. MiR-503 overexpression attenuates hPASMC proliferation and migration induced by hypoxia. A. miR-503 expression in PASMCs after hypoxia stimulation for 0, 6, 12 and 24 h was detected by qRT-PCR analysis. B. miR-503 mimics and NC mimics were transfected into hPASMCs, and the transfection efficiency was detected by qRT-PCR analysis. C. Cell proliferation was detected by MTT assay in hPASMCs. D. Cell migration was detected by wound healing assay in hPASMCs. The data are expressed as mean ± SD.

expression of miR-503 in hPASMCs was significantly enhanced after transfection of miR-503 mimics (**Figure 2B**).

The effect of miR-503 up-regulation on cell proliferation of hPASMCs was examined by MTT assay. The results showed that overexpression of miR-503 rescued the excessive proliferation of hypoxia-treated hPASMCs after 48 h of incubation (**Figure 2C**). The effect of miR-503 on cell migration of hPASMCs was determined by wound healing assay. As demonstrated in **Figure 2D**, the migratory ability of hPASMCs was dramatically increased under hypoxia treatment, whereas miR-503 up-regulation reserved the hypoxia-induced promotion of migration of hPASMCs.

MiR-503 overexpression arrests hPASMC cell cycle progression induced by hypoxia

Above findings showed miR-503 could inhibit the proliferation of hPASMCs, and then impact of miR-503 overexpression on cell cycle was further assessed by flow cytometry. As shown in **Figure 3A**, hypoxia treatment reduced the proportion of hPASMCs in G0/G1-phase, and the proportion of hPASMCs in S and G2/M phases markedly increased as compared to under normoxia. The deregulated cell cycle progression was obviously restored by transfection of miR-503 mimics.

Next, western blotting was used to investigate the expression of cell cycle relative proteins. As shown in **Figure 3B**, p27 expression was significantly decreased and Cyclin D1 was significantly increased in hypoxia-treated hPASMCs, and transfection of miR-503 mimics obviously reversed these effects.

MiR-503 overexpression restores hPASMC apoptosis inhibited by hypoxia

Flow cytometric analysis showed that the proportion of the cell population undergoing apoptosis was decreased in hPASMCs under hypoxia treatment, but this was reversed by transfection of miR-503 mimics in hPASMCs (**Figure 4A**).

To further explore the potential mechanism of hPASMC apoptosis, the expression of cell apoptosis relative proteins Bcl-2 and Bax was subsequently investigated by Western blotting. As exhibited in **Figure 4B**, hypoxia treatment increased expression of Bcl-2 and decreased expression of Bax, but these effects were largely eliminated by transfection of miR-503 mimics.

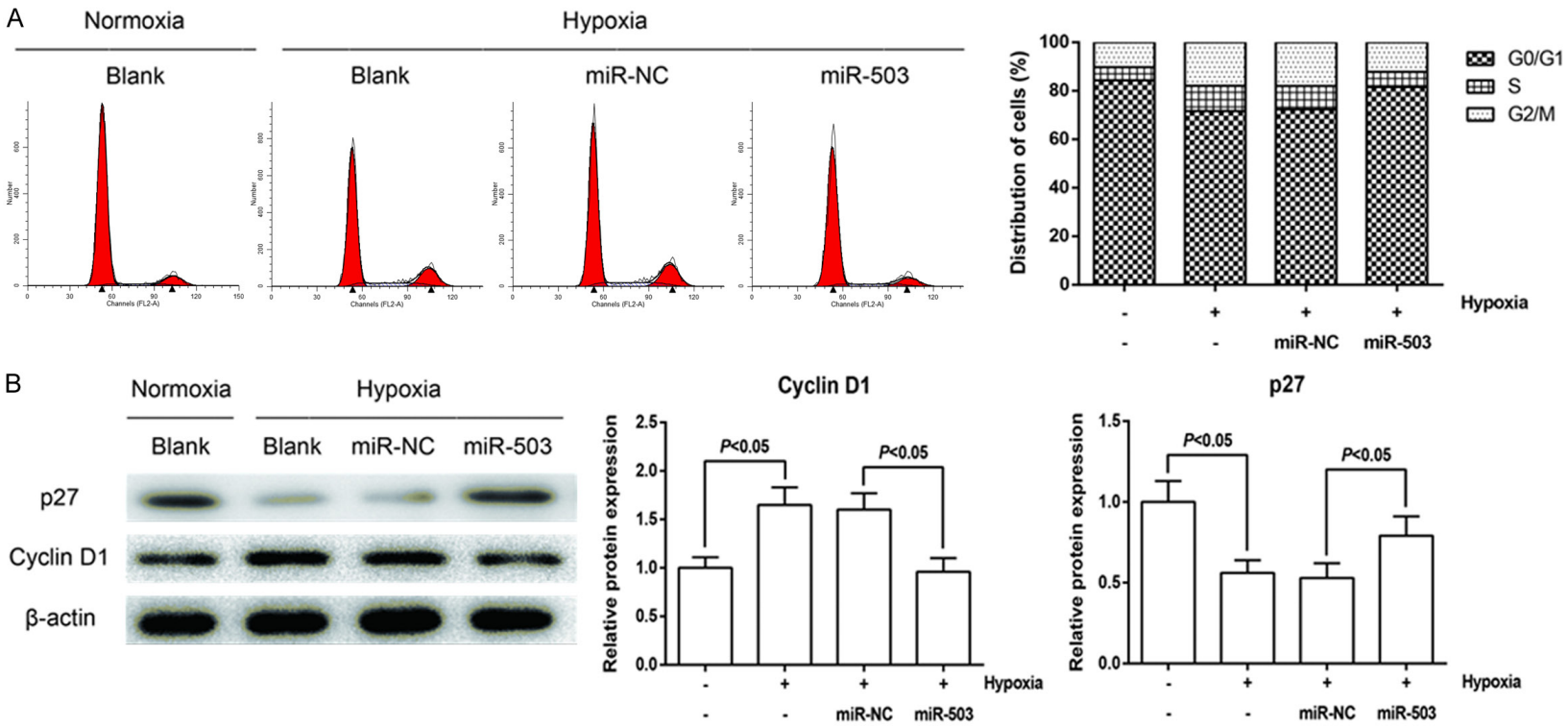

Figure 3. MiR-503 overexpression arrests hPASMC cell cycle progression induced by hypoxia. A. Cell cycle distribution of hPASMCs was detected by flow cytometry. B. p27 and Cyclin D1 expression in hPASMCs was detected by western blot analysis. The data are expressed as mean ± SD.

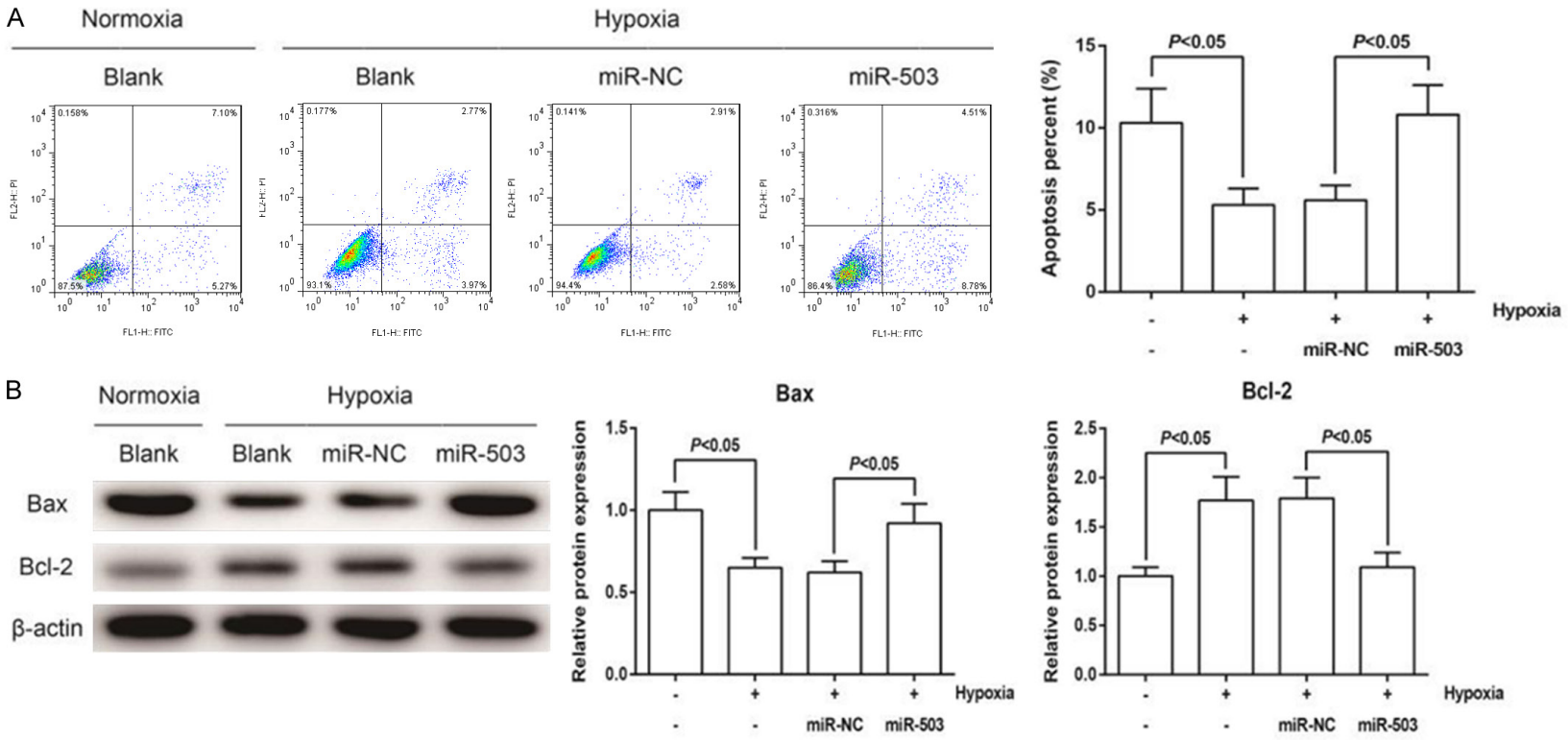

Figure 4. MiR-503 overexpression restores hPASMC apoptosis inhibited by hypoxia. A. Cell apoptosis of hPASMCs was detected by flow cytometry. B. Bcl-2 and Bax expression in hPASMCs was detected by western blot analysis. The data are expressed as mean ± SD.

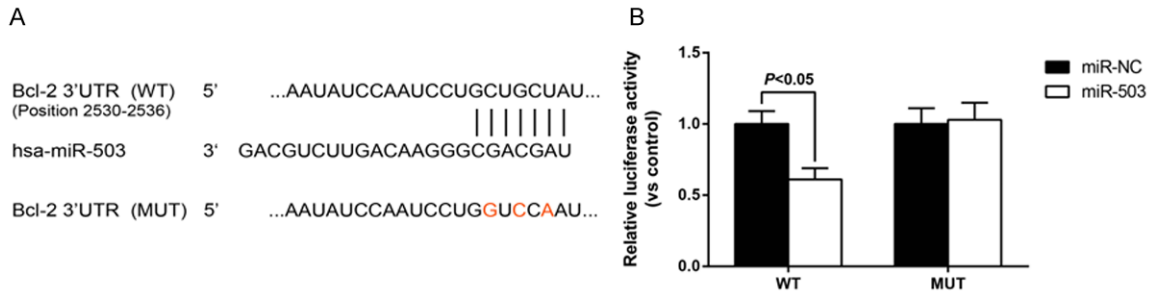

Figure 5. MiR-503 directly targets 3'-UTR of Bcl-2 in hPASMCs. A. Schematic representation of putative miR-503 targeting sites in the 3'-UTR of Bcl-2, and the generated mutant Bcl-2 3'-UTR. B. Relative luciferase activity of hPASMCs after co-transfection with wild-type (WT) or mutant (MUT) Bcl-2 3'-UTR reporter genes along with miR-503 or miR-NC. The data are expressed as mean ± SD.

MiR-503 directly targets 3'-UTR of Bcl-2 in hPASMCs

Given that the biological effects of altered miRNA expression rely on the significance of their target genes, we explored targets of miR-503 using the TargetScan bioinformatics algorithm (http://www.targetscan.org/). Our analysis revealed that Bcl-2 is a potential target of miR-503 based on putative target sequences of the Bcl-2 3'-UTR (**Figure 5A**). To confirm Bcl-2 as a direct target of miR-503, a dual-luciferase reporter assay was performed in hPASMCs. The two luciferase constructs containing the WT or MUT 3'-UTR of the Bcl-2 gene were then separately transfected into hPASMCs, together with either NC or miR-503. As a result, miR-503 dramatically reduced the luciferase activity of the WT-Bcl-2 3'UTR but not of the mutant in hPASMCs (**Figure 5B**). Thus, the complementary sequences on Bcl-2 3'-UTR were then a direct target of miR-503.

Discussion

The pathogenesis of PAH is a critical topic, but it is not yet fully understand. The present study is the first, to our knowledge, to identify decreased miR-503 expression in serum samples of treatment-naive IPAH patients and hypoxia-treated hPASMCs. Overexpression of miR-503 alleviated disease phenotypes in hypoxia-treated hPASMCs through regulating Bcl-2 expression.

MiRNAs have been investigated as a diagnostic and prognostic biomarker in numerous diseases. The extreme stability of circulating miRNAs in biological fluids and their resistance to various storage conditions make them good candidates for the development of minimally invasive biomarkers [11, 12]. Several studies examined circulating miRNAs and have demonstrated that they are correlated with PAH development and progression. For example, plasma miR-150 levels are decreased and correlate with survival in PAH patients [13]. In the present study, we found markedly decreased expression of miR-503 in IPAH patients compared to controls. Consistent with our findings, previous animal experiments showed that miR-503 was reduced in the lung and microdissected pulmonary artery of monocrotaline-induced PAH rats [14]. MiR-503 was also identified as an onco-suppressor in human tumorigenesis, including prostate cancer [15], osteosarcoma [16] and hepatocellular carcinoma [17]. In cardiovascular system, a recent study reported that overexpression of miR-503 increased the cellular proliferation and collagen production in mice neonatal cardiac fibroblasts [18]. The clinical significance of miR-503 promoted us to explore its underlying mechanisms in PAH.

The pulmonary vascular wall is made up of three resident cell types, including the endothelial (intima), smooth muscle (media) and fibroblast (adventitia) cells [19]. It has been recognized that hypoxia is a stimulus to enhance PASMC proliferation and migration capabilities, which play key roles in promoting vascular remodeling during the development of PAH [20]. Some miRNAs, such as miR-206 [21] and miR-322 [22], are involved in the hypoxia-induced excessive PASMC proliferation and migration. Our results showed that miR-503 expression was reduced in hypoxia-treated hPASMCs, and excessive hPASMC proliferation and migration caused by hypoxia was significantly attenuated by miR-503 overexpression.

Deregulated cell proliferation is mainly attributed to aberrant cell cycle regulation. Cyclin D1 is an important cell cycle gene that induces G1-to-S phase progression, leading to the promotion of cell proliferation [23]. Deregulated Cyclin D1 has been reported in PAH and has been implicated in PASMC proliferation [24, 25]. As a cyclin-dependent kinase inhibitor, p27 can cause cells in G1 arrest which contributes to the inhibition of PASMC proliferation [26, 27]. In this study, we found that miR-503 overexpression led to cell cycle arrest at G1 phase through increasing p27 expression and decreasing Cyclin D1 expression in hPASMCs.

The B-cell lymphoma protein-2 (Bcl-2) protein family, which included both pro-apoptotic (e.g., Bax) and anti-apoptotic (e.g., Bcl-2) members, play a critical role in the development of apoptosis [28]. Bcl-2 silencing can obviously attenuate hypoxia-triggered apoptosis resistance in pulmonary microvascular endothelial cells [29]. Several target genes regulated by miR-503 have been reported. For example, miR-503 regulates cisplatin resistance of human non-small cell lung cancer cells and gastric cancer cells through targeting Bcl-2 [30, 31]. In this article, we also found that ectopic expression of miR-503 caused a remarkable reduction in Bcl-2 protein level and decreased luciferase activity of the Bcl-2 promoter, indicating that Bcl-2 is a direct target of miR-503 in hPASMCs.

Taken together, the present study presents evidences that miR-503 plays a critical suppressive role in PAH by directly binding the Bcl-2 3'UTR that leads to down-regulation of Bcl-2 expression level. Our observation may provide clues that enhancing miR-503 expression might be a potential therapeutic target for PAH in the near future.

Disclosure of conflict of interest

None.

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