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# MicroRNA-204 as an Indicator of Severity of Pulmonary Hypertension in Children with Congenital Heart Disease Complicated with Pulmonary Hypertension

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Data Interpretation D  
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**Background:** The objective of this study was to investigate the changes and significance of microRNA-204 (miR-204) in children with congenital heart disease (CHD) complicated with pulmonary hypertension (PH).

**Material/Methods:** Fifty-two CHD patients with left-to-right shunt were divided into 3 groups according to preoperative pulmonary artery systolic pressure (PASP) detected by color Doppler echocardiography: a control group (PASP <30 mmHg), a mild PH group (PASP 30–49 mmHg), and a severe PH group (PASP >50 mmHg). Peripheral venous blood and supernatant were collected on an empty stomach at 1 h before surgery and 7 days after surgery. The expression of miR-204 in plasma was detected by RT-qPCR.

**Results:** One hour before surgery and 7 days after surgery, plasma miR-204 expression was at a higher level than that in the mild PH group and higher than in the severe PH group. miR-204 expression in children in each group showed a decreasing trend after surgery. The mild PH and severe PH groups had lower plasma miR-204 expression and PASP after surgery than before surgery. In the mild PH and severe PH groups, plasma miR-204 expression was negatively correlated with PASP. In all 52 cases, plasma miR-204 expression was negatively correlated with PASP.

**Conclusions:** The plasma miR-204 expression in CHD children with PH was negatively correlated with the degree of PH, suggesting miR-204 may be involved in PH development, and miR-204 expression may be an indicator of PH severity.

**MeSH Keywords:** **Heart Defects, Congenital • Pulmonary Heart Disease • Severity of Illness Index**

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## Background

Congenital heart disease (CHD) is the most common birth defect, affecting approximately 1% of live births, and it is reported that the morbidity of CHD is more than 10-fold higher in fetuses than in newborns [1]. Pulmonary hypertension (PH) is a common disease in CHD, which presents as a wide spectrum of disease entities with a defined mean pulmonary arterial pressure (mPAP)  $\geq 25$  mmHg at rest [2]. PH is a well-known complication that limits the survival and functional ability of patients with surgically unrepaired, palliated, and in some cases “repaired” CHD [3]. Focusing on pulmonary arterial hypertension (PAH) associated with CHD, the prevalence rate of PAH in adults was 5–10% and this had a great influence on mortality and morbidity, and resulted in increasing demand for life-long nursing care [4]. The etiology of PAH-associated CHD depends on the existence of defects, but it is also believed that environmental factors and genetics or epigenetics play a role [4]. PH in CHD is commonly secondary to left-to-right shunt defects or left heart obstructive disease resulting in postcapillary hypertension [5]. The criterion standard for measuring PH is right cardiac catheterization, but this is an invasive method with an inherent risk of complications [6]. The prognosis of PAH is predicted by markers of heart failure, plasma concentration of brain natriuretic peptide (BNP) and bilirubin, but the prediction of survival is not applicable for all [7]. Thus, clinical practice needs a new severity indicator effective for all PH patients.

In recent years, microRNA (miR), as a conserved, non-protein-encoded RNA molecule, has been considered as an important mediator of many genes in cardiovascular diseases [8]. Because of its multidirectional vascular function, miR can coordinate many disease pathways in pulmonary vessels, but their importance in PH is only beginning to emerge [9]. It is reported that miR-204 plays important roles in smooth muscle cell calcification and endoplasmic reticulum stress response in trabecular meshwork cells [10,11]. In addition, recent reports have shown that miR-204 is equally important in tumorigenesis, including the regulation of carcinogenesis in peripheral nerve sheath tumors, and migration and invasion of endometrial cancer cell lines [12,13]. Furthermore, overexpression of miR-204 can obviously promote the proliferation of cardiomyocyte in both neonatal and adult rat cardiomyocytes [14]. However, very little is known about the mechanism by which miR-204 regulates PH in CHD. Thus, in our study, we selected 52 children with CHD with left-to-right shunt for assessing the plasma miR-204 expression to investigate the correlation between miR-204 and PH degree.

## Material and Methods

### Study subjects

Fifty-two children with CHD with left-to-right shunt were selected from the Department of Cardiac Surgery of Guizhou Provincial People's Hospital from March 2017 to September 2018. Among them, 21 were males and 31 were females (aged from 3 months to 10 years), including 20 cases of simple ventricular septal defect (VSD), 11 cases of simple atrial septal defect (ASD), 8 cases of VSD with ASD, 7 cases of patent ductus arteriosus (PDA), 4 cases of VSD with PDA, and 2 cases of ASD with PDA. They were diagnosed by color echocardiography. Patients with the following conditions were excluded: heart failure, thyroid diseases, hematological diseases, autoimmune diseases, HIV infection, severe liver and renal insufficiency, and malignant tumors. The study was approved and supervised by the Ethics Committee of Guizhou Provincial People's Hospital. All subjects or their families signed the informed consent.

### Grouping

According to the pulmonary artery systolic pressure (PASP) measured by echocardiography [15], 52 children with CHD were divided into: a control group of 13 cases (6 males and 7 females, mean age of  $5.15 \pm 2.28$ ) without PH (PASP  $< 30$  mmHg); a mild PH group with 18 cases (male 7 cases and female 11 cases, mean age of  $4.74 \pm 1.93$ ) with mild PH (PASP 30–49 mmHg); and a severe PH group with 21 cases (male 8 cases and female 13 cases, mean age of  $5.50 \pm 2.06$ ) with moderate and severe PH (PASP  $> 50$  mmHg). There was no significant difference in age or sex among these 3 groups.

### Hemodynamic measurements

A cardiovascular detector (VBP-10, Doppler) was used to measure the preoperative cardiac index, heart rate, and stroke volume.

### Measurement of PASP

Color Doppler echocardiography was used to estimate PASP according to tricuspid regurgitation pressure difference [16]. Right ventricular systolic pressure (RVSP) was estimated by Doppler ultrasound measurement of right ventricular and right atrial pressure difference (i.e., tricuspid valve pressure difference):  $RVSP = \text{right atrial pressure (PAP)} + \text{tricuspid transvalvular pressure } (\Delta P)$ . According to the modified Bemoulli equation:  $\Delta P = 4V^2$ ,  $V$  is maximum tricuspid regurgitation velocity (m/s). Right atrial pressure can be estimated by the width of the inferior vena cava or by observing the pulsatile amplitude of the jugular vein by physical examination. Generally,

right atrial pressure is calculated by 5 mmHg, so as to indirectly judge PASP [17]. The echocardiographic examination of enrolled patients was performed by the same experienced ultrasound physician on the same ultrasound instrument, and the images were collected strictly according to the operation manual. The obtained images were measured independently by 2 physicians.

### Operation and management after surgery

All 52 patients were treated with conventional corrective surgery, combined intravenous and inhalation anesthesia, and cardiopulmonary bypass (CPB) and equipment. The parameters were controlled at normal level. There was no significant difference in CPB and ascending aorta occlusion time among groups. Five patients with PDA underwent off-pump surgery, and the rest were operated on under general anesthesia and hypothermic CPB. Respirator-assisted breathing was performed after surgery (11.9±3.8 h), and hemodynamic and stable internal environment were maintained. All patients recovered and were discharged from the hospital.

### Sample collection and processing

Radial artery blood (5 mL) from each patient was collected 1 h before surgery and 7 days after surgery, and was injected into test tubes containing heparin sodium and shaken well. After that, the blood was placed in a refrigerator at 4°C for 2 h and then centrifuged at 3000 rpm and 4°C for 15 min. The serum was gathered and placed in an EP tube without RNA enzyme. The blood was kept at -80°C to avoid repeated freezing and thawing.

### Quantitative analysis of miR-204 by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The serum and Trizol (Invitrogen, USA) in equal volumes were collected by in an EP tube (2 mL), and miR was extracted according to miRNeasy Mini Kit (QIAGEN, Germany) instructions. The concentration and purity of RNA expressed as OD260/280 were determined by use of a NanoDrop 1000 micronucleic acid analyzer (Eppendorf, Germany). The results showed the RNA purity was over 1.8 and the concentration was above 1.5 µg/µL, and was suitable for use in subsequent experiments. The total RNA was reversely transcribed into cDNA of specific miR with the reaction at 16°C for 15 min, at 42°C for 1 h, and finally at 85°C for 5 min. The synthesized cDNA was kept at -20°C. miR-204 expression was detected with the TaqMan microRNA Assay (Applied Biosystems). The miR-204 quantitative primer was 5'-CTCGCTTCGGCAGCAC-3' (upstream primer) and 5'-ACCGTGCTGGAGTCGGCAATT-3' (downstream primer), while the U6 quantitative primer was 5'-GTGCGTGCTGGAGTCG-3' (upstream primer) and

5'-AACGCTTCACGAATTTGCGT-3' (downstream primer). The reaction system included 10×PCR buffer solution (2 µL), 25 mmol MgCl<sub>2</sub> (1.2 µL), 10 mmol dNTP (0.4 µL), primers (1 µL), Taq enzyme (0.4 µL), template cDNA (1 µL), and DEPC water (14 µL). Reaction conditions were pre-denaturation at 94°C for 5 min, and 40 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 40 s. The qPCR was performed in ABI StepOnePlus RT-PCR system, while data were analyzed by StepOne software (Applied Biosystems). Finally, relative expression was calculated by 2<sup>-ΔΔCt</sup> method.

### Statistical analysis

SPSS 19.0 (IBM Corp. Armonk, NY, USA) was used for data analysis and measurement data are presented in means±standard deviation. The homogeneity of variance test was conducted before data analysis. The single-factor analysis of variance (one-way ANOVA) method was used in multi-group comparisons, and pairwise comparisons of multi-groups/mean values were conducted using the LSD-t test. *P*<0.05 showed the difference was statistically significant.

## Results

### Comparison of baseline data of clinical samples

Fifty-two children with left-to-right shunt CHD admitted from the Department of Cardiac Surgery of Guizhou Provincial People's Hospital from March 2017 to September 2018 were enrolled, including 21 males and 31 females aged from 3 months to 10 years. The more detailed demographic characteristics of samples are shown in Table 1. There were no statistically significant differences in sex, age, heart index, heart rate, or stroke volume among these 3 groups (all *P*>0.05).

### Comparison of plasma miR-204 expression before and after surgery

As shown in Figure 1A, the plasma miR-204 expression in the control group before surgery was at a higher level than in the mild PH group (*P*<0.05) and in the severe PH group (*P*<0.01).

As shown in Figure 1B, the plasma miR-204 expression in the control group after surgery was at a higher level than in the mild PH group (*P*<0.001) and in the severe PH group (*P*<0.01).

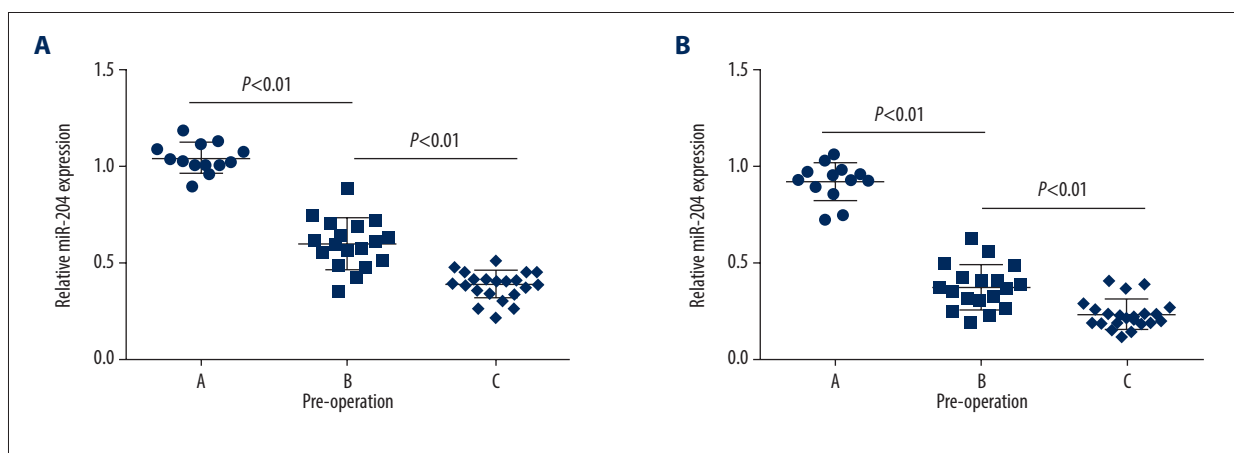
### Intragroup comparison of plasma miR-204 expression before and after surgery

No significant difference was found in plasma miR-204 expression in the control group before and after surgery (*P*>0.05). The plasma miR-204 expression was lower after surgery than

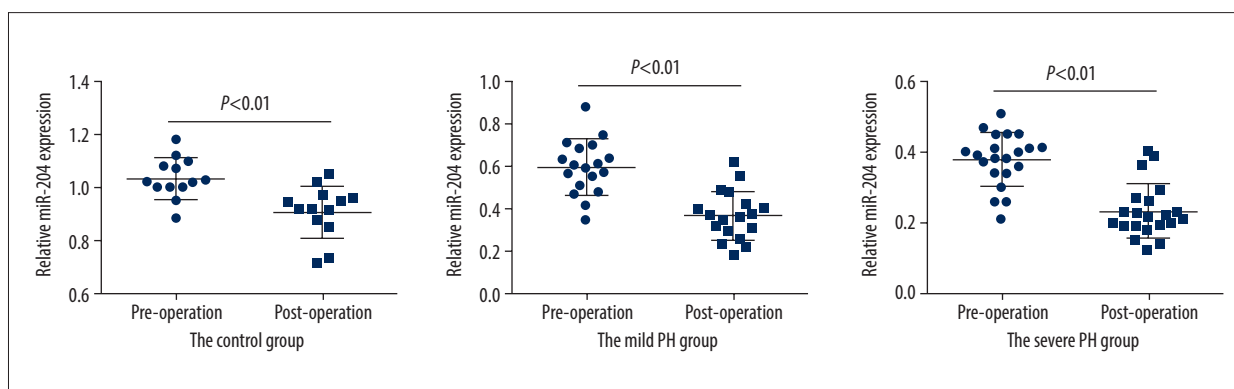
**Table 1.** Demographic characteristics of the respondents.

	Control group (n=13)	Mild PH group (n=18)	Severe PH group (n=21)	*p value	**p value	***p value
Gender						
Male	6	7	8	0.349	0.257	0.686
Female	7	11	13			
Age	5.150±2.280	4.740±1.930	5.500±2.060	0.592	0.647	0.245
Cardiac Index L/(min×m <sup>2</sup> )	2.010±0.130	1.950±0.420	1.880±0.270	0.624	0.116	0.534
Heart rate (min <sup>-1</sup> )	139.520±13.120	148.480±22.030	151.040±9.830	0.202	0.073	0.705
Stroke volume (mL)	1.660±0.240	1.590±0.190	1.510±0.310	0.372	0.064	0.223

\* Represented Control group vs. mild-PH group. \*\* Represented Control group vs. severe-PH group. \*\*\* Represented mild-PH group vs. severe-PH group. Chi-square test was utilized to determine statistical significance. For Control group, N=13, while in mild-PH group and severe-PH group, N=18 and 21, respectively.



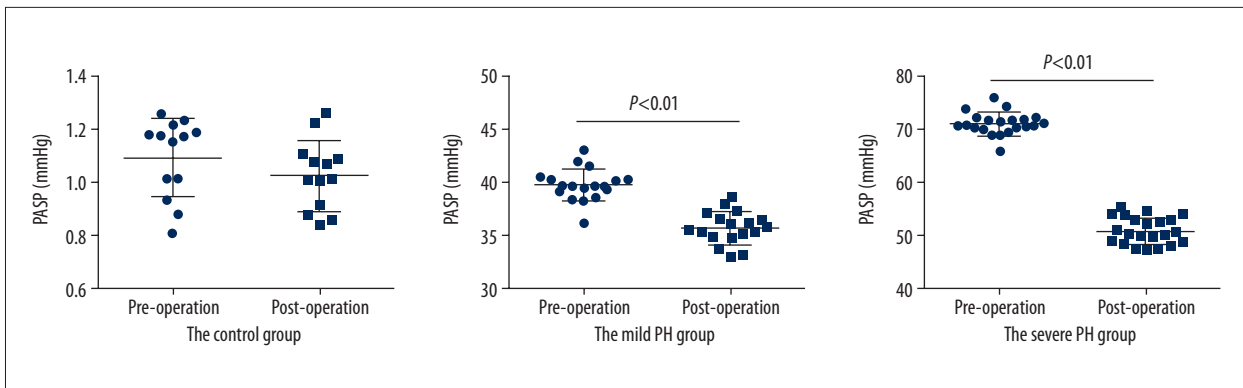
**Figure 1.** Comparison of plasma miR-204 expression before and after surgery among the 3 groups. (A) Comparison of plasma miR-204 expression before surgery; (B) comparison of plasma miR-204 expression after surgery.



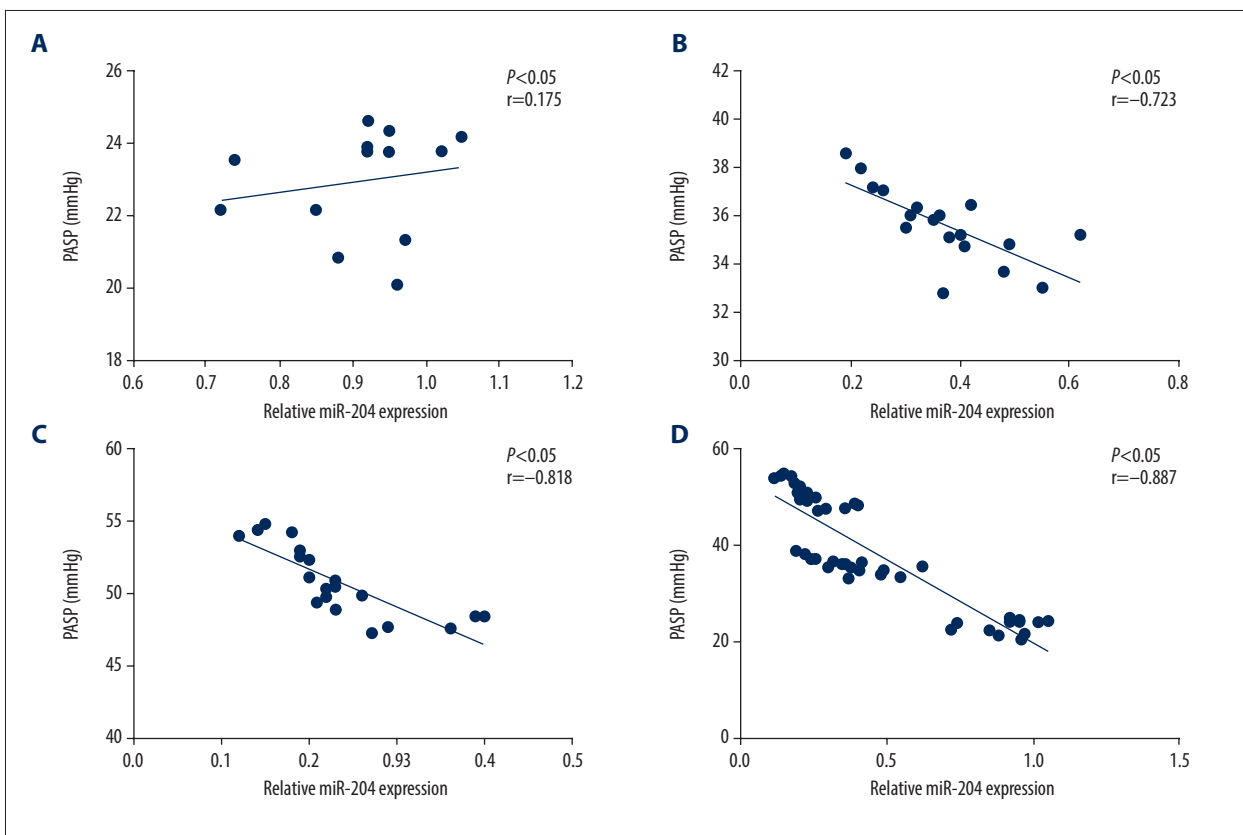
**Figure 2.** Comparison of plasma miR-204 expression before and after surgery in intragroup.

before surgery in the mild PH group ( $P<0.05$ ) and in the severe PH group ( $P<0.01$ ) (Figure 2). This suggests that the plasma miR-204 expression decreased with the increase of

PH. The decrease of PH and the decrease of miR-204 content in the same group after surgery further confirmed the relationship between miR-204 and PH.



**Figure 3.** Comparison of PASP before and after surgery in intragroup.



**Figure 4.** Negative correlation was found between plasma miR-204 expression and PASP after surgery. (A) Correlation analysis between plasma miR-204 expression and PASP in the control group; (B) correlation analysis between plasma miR-204 expression and PASP in the mild PH group; (C) correlation analysis between plasma miR-204 expression and PASP in the severe PH group; (D) correlation analysis between plasma miR-204 expression and PASP in all 45 children patients.

### Comparison of PASP before and after surgery in intragroup

Reexamination of echocardiography was performed in children 7 days after surgery. No significant difference in PASP was found in the control group before and after surgery ( $P > 0.05$ ). PASP was lower after surgery than before surgery in the mild group ( $P < 0.05$ ) and also in the severe PH group ( $P < 0.01$ ) (Figure 3).

### Correlation analysis between plasma miR-204 expression and PASP

We analyzed the correlation between plasma miR-204 expression and PASP in 3 groups of children after surgery. The results showed that there was no correlation between plasma miR-204 expression and PASP in the control group ( $r = 0.175$ ,  $P = 0.568$ , Figure 4A). In the mild PH group, a negative correlation was

found between plasma miR-204 expression and PASP ( $r=-0.723$ ,  $P=0.006$ , Figure 4B). In the severe PH group, a negative correlation was found between plasma miR-204 expression and PASP ( $r=-0.818$ ,  $P<0.001$ , Figure 4C). There was also a negative correlation between plasma miR-204 expression and PASP in all 52 children patients ( $r=-0.887$ ,  $P<0.001$ , Figure 4D).

## Discussion

miRNAs have been demonstrated to be involved in human diseases, including cardiovascular diseases, and a previous study has reported that several miRNAs were aberrantly expressed in PAH [18]. In the present study, the plasma miR-204 expression in CHD complicated with PH was investigated with the involvement of 52 CHD with left-to-right shunt. We found that plasma miR-204 expression in CHD children with PH was negatively correlated with PH degree, suggesting that miR-204 is involved in the development of PH, and miR-204 expression may be an indicator of the severity of PH.

We firstly detected the plasma miR-204 expression before and after surgery and the plasma miR-204 expression before and after surgery and performed intragroup comparison. We found that with the increase of PH, the plasma miR-204 expression decreased. miRNAs are widely used as biomarkers for heart failure, myocardial infarction, and some other heart diseases [19–21]. The presence of cardiovascular miR molecules in circulating nucleic acids in patients with cardiovascular disease suggests that this molecule may be an effective noninvasive diagnostic tool [22]. In addition, Rabinovitch et al. proved the molecular pathogenesis of PAH with the involvement of miRNAs [23]. In line with our study, Courboulin et al. also demonstrated that miR-204 was underexpressed in PAH [19]. Potus et al. reported miR-204 expression was decreased in PAH human lungs and was correlated with PAH severity [24]. A previous study found a negative correlation between PAH and miR-204, and suggested miR-204 is a good clinical serological indicator for diagnosis of patients with PAH due to CHD [25]. Thus, we concluded miR-204 could act as a diagnostic tool, a prognostic predictor, or a monitor of response to PH therapy.

Reexamination of echocardiography was performed in children 7 days after surgery, and the results revealed that the PASP was decreased after surgery in patients with CHD complicated with PH. A negative correlation was found between plasma miR-204 expression and PASP, suggesting the ability of miR-204 to predict the severity of PH. PAH is one of the

most serious complications of advanced CHD, characterized by vascular proliferation and remodeling of pulmonary arterioles, with high disability and mortality rates [26]. Currently, echocardiography is usually applied for suspected PAH cases; however, echocardiographic estimation of the PAP has obvious errors and low accuracy, although the velocity of tricuspid regurgitation is closely related to its pressure gradient [27]. Interestingly, downregulated miR-204 has been found in many kinds of tumors, including non-small cell lung cancer, gastric cancer, bladder cancer, and head and neck cancer, and it has been proved that downregulation of miR-204 results in tumor growth [28–30]. In addition, miR-204 is reduced in plexiform vasculopathy of severe PAH in humans, and is predicted to be a disease-modified miR in PAH and is established as a key factor in hypoxia-induced pulmonary hypertension in mice [9,31,32]. The decrease of miR-204 expression was also related to decreased apoptosis, increased cell proliferation, and membrane depolarization, which were the most common changes in PAH- pulmonary artery smooth muscle cells [11,33,34]. miR-204 expression was also reported to be decreased in PAH and was correlated with PAH severity, which was consistent with our conclusion [24].

## Conclusions

We provided significant evidence that the plasma miR-204 expression in CHD children with PH was negatively correlated with the degree of PH, suggesting that miR-204 may be involved in the development of PH, and miR-204 expression may be one of the indicators to judge the severity of PH and to monitor response to PH therapy. However, in this study, the severity of PH was graded using an echocardiographic recording of PASP, which is not the criterion standard for the diagnosis of PH. Thus, 2 of the 3 groups in this study were diagnosed using a sub-optimal diagnostic test. This is ethically justified due to the invasiveness of right heart catheterization. In the future, we will perform follow-up of the study participants, investigate the relationship between their PH status (e.g., symptoms, signs, and echocardiographic data) and their miR-204 levels at follow-up visits, and provide a more detailed stratification of changes in circulating miR-204 based on the type of congenital heart disease and surgical intervention.

## Conflict of interest

None.

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