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RESEARCH ARTICLE

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Expression and clinical significance of circular RNA hsa_circ_0003416 in pediatric pulmonary arterial hypertension associated with congenital heart disease

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Abstract

Background: Circular RNAs (circRNAs) have been found to be involved in the development of pulmonary arterial hypertension (PAH). However, their diagnostic value in pediatric PAH remains unclear. This study aimed to examine the characteristic expression of the circRNA hsa_circ_0003416 in the plasma of children with PAH caused by congenital heart disease (CHD); the potential of hsa_circ_0003416 as a diagnostic biomarker was also investigated.

Methods: The plasma expression levels of hsa_circ_0003416 were determined via quantitative reverse transcription–polymerase chain reaction in 50 CHD patients, 50 PAH patients, and 20 healthy subjects; the associations between hsa_circ_0003416 levels and clinical data were analyzed thereafter. Receiver operating characteristic curves were employed to determine the diagnostic capacity of this circRNA.

Results: Expression levels of hsa_circ_0003416 in plasma were lower in the PAH-CHD group than in the CHD and healthy control groups ($p = 0.009$ vs. healthy control group, $p = 0.026$ vs. CHD group). Moreover, hsa_circ_0003416 was found to be negatively associated with B-type natriuretic peptide ($r = -0.342$, $p = 0.013$). In addition, the area under the curve of hsa_circ_0003416 levels in plasma was 0.721 (95% confidence intervals = 0.585–0.857, $p = 0.004$), suggesting that it has a promising diagnostic value.

Conclusions: Overall, hsa_circ_0003416 was found to be significantly downregulated in children with PAH-CHD and to be potent as a biomarker for PAH-CHD diagnosis.

KEYWORDS
biomarker, circular RNA, congenital heart disease, pediatric patients, pulmonary arterial hypertension

1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a devastating vascular disorder characterized by an increase in pulmonary vascular resistance (PVR); this eventually evolves into right heart dysfunction and can potentially result in death.[1,2] Data reported by China registry studies have indicated that the most frequent cause of PAH in children is congenital heart disease (CHD),[3] with approximately 5%–10% of CHD patients eventually progressing to develop varying degrees of PAH.[4] Due to the lack of specific symptoms at an early stage, the diagnosis of PAH associated with CHD (PAH-CHD) is often delayed. Patients with CHD have been reported to be diagnosed with PAH approximately 6 years after symptom onset[5]; occurrence of PAH increases the mortality rate of patients with CHD more than twofold compared with that of patients without PAH.[6] Despite great advancements in treatment, the prognosis of children with PAH-CHD remains poor.[7] Thus, methods to aid the early diagnosis and effective treatment of PAH-CHD are urgently required. Currently, cardiac catheterization remains the gold standard for PAH diagnosis, and it can directly assess pulmonary hemodynamics and perform vasoreactivity test.[8] However, this approach may increase the risk of complications, such as puncture injury, arrhythmias, hypertensive crisis, pulmonary embolism, and even death; therefore, it is not suitable for repeated evaluation.[8] Echocardiography is a non-invasive and widely available tool for patients with PAH-CHD. However, it is relatively expensive, and its accuracy is influenced by many factors, such as the experience of operator and quality of the equipment. Currently, a specific, inexpensive, and non-invasive method for screening PAH-CHD is lacking[9]; therefore, identification of effective non-invasive biomarkers for clinical practice is imperative.

Circular ribonucleic acids (circRNAs) are an emerging type of endogenous RNAs that are produced by the back-splicing of pre-messenger RNA.[10] Previously, circRNAs were considered to be non-coding RNAs. However, recent studies have shown some of them to have translational function.[11,12] In contrast to traditional linear RNAs, circRNAs form covalent closed-loop structures without a 5′-cap or 3′-polyadenylate tail.[13] Their unique circular structures protect them from degradation by RNA exonuclease, thereby rendering them stable and abundant in tissues and body fluid.[14,15] This makes them promising clinical biomarkers for diseases. CircRNAs can regulate gene expression at the transcriptional and post-transcriptional levels by interacting with microRNA (miRNA) or RNA-binding proteins, and can participate in various biological process.[14,16] They have also been widely implicated in a variety of diseases, including cardiovascular diseases, diabetes, cancer, and nervous system diseases.[17–20] Aberrant expression of circRNAs may be involved in the pathogenesis of PAH.[21–24] However, the potential functions of most circRNAs in PAH have not yet been clarified.

The present study aimed to assess the potential of plasma circRNAs in aiding the diagnosis of PAH-CHD in children. The circRNA hsa_circ_0003416 was selected as a research target based on previous microarray data (GSE171827 in the Gene Expression Omnibus database); it had previously been found to be one of the most downregulated circRNAs in the plasma of PAH-CHD children. This study, therefore, examined the characteristic expressions of hsa_circ_0003416 in a larger sample size and analyzed its clinical value, with the aim of determining whether hsa_circ_0003416 could possibly serve as a biomarker for PAH-CHD diagnosis.

2 | MATERIALS AND METHODS

2.1 | Participants and plasma samples

This study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University (No. 2021; KY-E-156). A total of 100 CHD children were recruited for this study at the Department of Pediatrics, First Affiliated Hospital of Guangxi Medical University, between January 2020 and March 2021. Parental informed consent was obtained for this study for each subject. The inclusion criteria were as follows: (1) age <14 years and (2) patient underwent cardiac catheterization before intervention. Patients with severe infections, tumors, severe cardiopulmonary dysfunctions, diabetes mellitus, systemic hypertension, cardiomyopathies, liver dysfunctions, or renal failure were excluded. PAH was diagnosed via cardiac catheterization. CHD patients were classified into either the PAH-CHD group (mean pulmonary artery pressure [mPAP] >20 mmHg, $n = 50$) or the CHD group (mPAP ≤20 mmHg, $n = 50$) based on their mPAP.[25] Detailed health history, demographic data, laboratory examination, echocardiography, and cardiac catheterization findings were collected. In addition, 20 healthy subjects were recruited as controls. Venous blood (3–5 ml) was drawn from each subject and then centrifuged (1000 *g* for 15 min at 4°C) to separate the plasma. The samples were then kept at −80°C until further use.

2.2 | RNA and DNA extraction

Total RNA was isolated from plasma samples using the BIOG cfRNA Easy Kit (Changzhou Baidai Biotechnology Co., Ltd.). It was then purified with an RNA purification and concentration kit (Tianmo Biotech) following the manufacturer's protocol. Genomic DNA (gDNA) was extracted using the TIANamp Genomic DNA Kit (Tiangen Biotech). Purity and concentration of RNA and DNA were quantified by spectrophotometry using a NanoDrop 2000 (Thermo Fisher Scientific). RNA/DNA samples with an A_{260}/A_{280} ratio of 1.8–2.2 were used for subsequent experiments.

2.3 | Quantitative reverse transcription–polymerase chain reaction

RNA was reverse transcribed into complementary DNA (cDNA) using the GoScript™ Reverse Transcription System Protocol

TABLE 1 Primer sequences

Gene name	Primer types	5′–3′	Product length (bp)
hsa_circ_0003416	Divergent F	CCCCTTTCACATCAAAGAAC	114
	Divergent R	ATTTAAACTTGATCCAACATGC	
	Convergent F	GTTTAAATGACTGTGCTGCCCCTTTC	99
	Convergent R	TTCCCTGCCAGCCAGATAGATAGAC	
GAPDH	Convergent F	CAGGAGGCATTGCTGATGAT	138
	Convergent R	GAAGGCTGGGGCTCATTT	

Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(Promega). A quantitative reverse transcription–polymerase chain reaction (qRT-PCR) experiment was conducted using the ChamQ™ Universal SYBR qPCR Master mix (Vazyme Biotech) with a 7500 Real-Time PCR System (Applied Biosystems). The qRT-PCR reaction mixture (20 μl) contained 0.4 μl each of 10 μM forward and reverse primers, 10 μl of 2× ChamQ Universal SYBR qPCR Master mix, 2 μl of cDNA, and 7.2 μl of ribonuclease (RNase)-free water. The amplification conditions were as follows: 30 s at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Glyceraldehyde 3-phosphate dehydrogenase was selected as the internal reference, and relative RNA expression was determined using the $2^{-\Delta\Delta Ct}$ method. Primers (Table 1) were synthesized by Sangon Biotech. The specificity of primers was confirmed using melting curve, agarose gel electrophoresis, and product sequencing analyses.

2.4 | RT-PCR

To verify the circular characteristics of hsa_circ_0003416, RT-PCR was performed with divergent and convergent primers using 2× SanTaq PCR Mix (Sangon Biotech), following the manufacturer's protocols. Both cDNA and gDNA were used as templates. The RT-PCR mix (50 μl) consisted of 2 μl each of 10 μM forward and reverse primers, 25 μl of 2× SanTaq PCR Mix, 2 μl of cDNA/gDNA, and 19 μl of RNase-free water. The PCR reaction conditions were set according to the manufacturer's recommendations. The amplification products were further verified by 1% agarose gel electrophoresis.

2.5 | Bioinformatics analysis

The circMIR software was used to predict circRNA-miRNA interactions, based on the RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) and miRanda (http://www.microrna.org/) databases. The miRWalk database (http://zmf.umm.uniheidelberg.de/apps/zmf/mirwalk/) was used to predict the target genes of miRNAs targeting hsa_circ_0003416, and the top 30 genes of each miRNA were selected. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted using the DAVID database (http://david.abcc.ncifcrf.gov), based on the selected target genes. The GO terms and KEGG pathways with p-value <0.05 were considered significantly enriched.

2.6 | Statistical analysis

SPSS 25.0 software (IBM) was applied for data processing. Categorical variables are presented herein as frequencies, and quantitative variables are presented either as means with standard deviation or as medians with interquartile range. Fisher's exact or chi-square test was used to test the categorical variables. Data normality was tested using the Shapiro–Wilk normality test. If a quantitative variable conformed to a normal distribution, a Student's *t*-test or analysis of variance was applied; otherwise, the Mann–Whitney *U*-test or the Kruskal–Wallis H-test was used. Receiver operating characteristic (ROC) curves were established using SPSS 25.0 to assess the diagnostic values. Correlation was calculated using Spearman correlation analysis. The predictors of PAH-CHD were identified by logistic regression analysis. The baseline characteristics, including age, gender, body mass index (BMI), and hsa_circ_0003416 and B-type natriuretic peptide (BNP) levels, were included in the multivariable regression analysis model as independent variables, whereas the presence of PAH-CHD was considered a dependent variable. $p < 0.05$ (bilateral) indicated statistical difference.

3 | RESULTS

3.1 | Subject characteristics

Among the 120 subjects involved in this study (50 PAH-CHD patients, 50 CHD patients, and 20 healthy subjects), sex, age, and BMI did not differ significantly across the groups ($p > 0.05$; Table 2). As shown in Table 3, PAH-CHD patients had a higher cardiothoracic ratio, systolic pulmonary arterial pressure (sPAP), mPAP, diastolic pulmonary arterial pressure (dPAP), PVR, pulmonary-to-systemic flow ratio (QP/QS), and BNP and creatine kinase-MB (CK-MB) levels than CHD patients ($p < 0.05$) while displaying a lower systolic blood pressure (SBP), diastolic blood pressure (DBP), ejection fraction (EF), and cardiac index (CI); no difference was noted in the left ventricular end-diastolic diameter (LVEDd), mean right atrial pressure, and CHD types between the two groups ($p > 0.05$).

TABLE 2 Baseline characteristics of subjects

Parameter	PAH-CHD group ($n = 50$)	CHD group ($n = 50$)	Healthy group ($n = 20$)	p-Value
Gender (n)				
Male	24	19	13	0.12
Female	26	31	7	
Age (years)				
≤5	41	45	13	0.065
>5	9	5	7	
BMI (kg m^{-2})				
≤15	28	37	10	0.08
>15	22	13	10	

Note: Values expressed show frequency (n); chi-square or Fisher's exact test was used for analysis.

Abbreviations: BMI, body mass index; CHD, congenital heart disease; PAH-CHD, pulmonary arterial hypertension associated with congenital heart disease.

TABLE 3 Clinical characteristics of the patients

Variable	CHD group	PAH-CHD group	P-value
CHD type (n)			
VSD	30	31	0.652
ASD	13	8	
PDA	6	9	
TAPVR	1	1	
AORPA	0	1	
SBP (mmHg)	107.78 ± 10.26	101.39 ± 14.17	0.012
DBP (mmHg)	67.40 ± 8.86	60.55 ± 11.84	0.002
EF (%)	72.48 ± 5.39	69.99 ± 6.15	0.034
LVEDd (mm)	35.16 ± 4.09	34.30 ± 5.90	0.399
Cardiothoracic ratio	0.52 ± 0.05	0.61 ± 0.05	<0.001
sPAP (mmHg)	25.30 ± 4.38	65.94 ± 14.77	<0.001
mPAP (mmHg)	16.00 ± 2.60	42.80 ± 11.62	<0.001
dPAP (mmHg)	12.60 ± 2.96	30.94 ± 11.25	<0.001
mRAP (mmHg)	5.00 (4.00–6.00)	5.50 (3.00–8.00)	0.058
PVR (WU)	1.71 (1.13–2.29)	4.26 (2.02–6.39)	<0.001
QP/QS	1.70 (1.50–2.20)	4.80 (3.50–6.20)	<0.001
CI (L $min^{-1}m^{-2}$)	8.00 (6.30–9.97)	6.00 (4.29–8.77)	0.001
BNP (pg ml^{-1})	129.25 (61.49–226.50)	1064.50 (288.35–3945.25)	0.001
CK-MB (U L^{-1})	21.00 (17.00–25.50)	24.00 (19.00–31.00)	0.016
hsa_circ_0003416	1.02 (0.78–1.38)	0.90 (0.81–1.04)	0.026

Note: The number of CHD types are expressed as frequencies (n); Fisher's exact test was used for analysis. The SBP, DBP, EF, LVEDd, cardiothoracic ratio, sPAP, mPAP, and dPAP conformed to normal distributions and are presented as means ± standard deviation; Student's t-test was used for analysis. mRAP, PVR, QP/QS, CI, BNP, CK-MB, and hsa_circ_0003416 did not conform to normal distribution; they are presented as medians with interquartile range; Mann–Whitney U-test was used for analysis.

Abbreviations: AORPA, anomalous origin of the right pulmonary artery from the ascending aorta; ASD, atrial septal defect; BNP, B-type natriuretic peptide; CHD, congenital heart disease; CI, cardiac index; CK-MB, creatine kinase-MB; DBP, diastolic blood pressure; dPAP, diastolic pulmonary arterial pressure; EF, ejection fraction; LVEDd, left ventricular end-diastolic diameter; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PAH-CHD, pulmonary arterial hypertension associated with congenital heart disease; PDA, patent ductus arteriosus; PVR, pulmonary vascular resistance; QP/QS, pulmonary to systemic flow ratio; SBP, systolic blood pressure; sPAP, systolic pulmonary arterial pressure; TAPVR, total anomalous pulmonary venous return; VSD, ventricular septal defect.

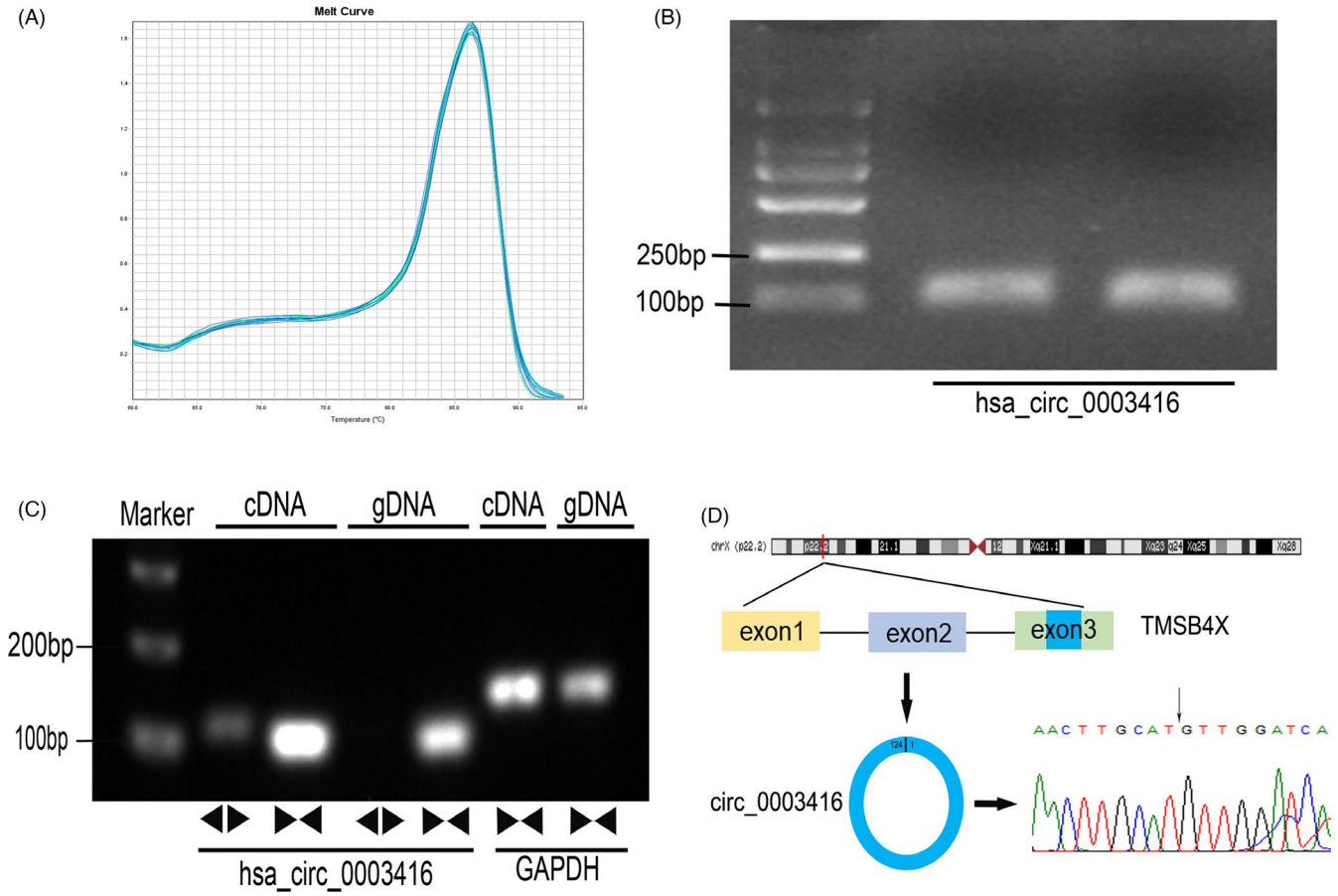

FIGURE 1 Validation of hsa_circ_0003416. (A) Melting curve. (B) Gel electrophoresis of the polymerase chain reaction (PCR) product. (C) Divergent primers (◄►) for detecting hsa_circ_0003416 in complementary DNA (cDNA) but not in genomic DNA (gDNA). (d) Sanger sequencing of the back-spliced junction (↓) of hsa_circ_0003416

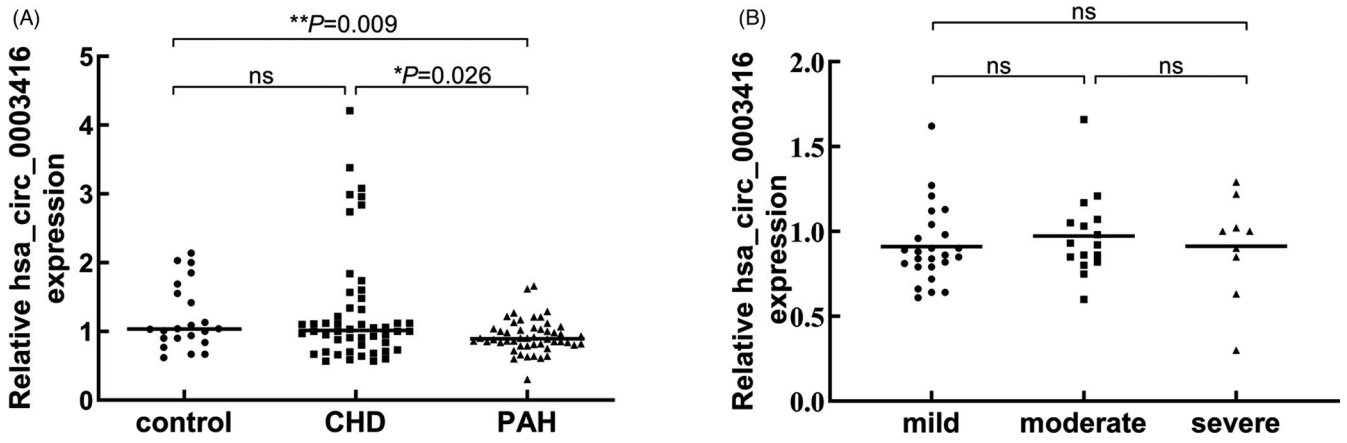

FIGURE 2 Relative hsa_circ_0003416 expression levels. (A) Relative hsa_circ_0003416 expression levels in the plasma of PAH-CHD patients, CHD patients, and healthy controls. (B) Relative hsa_circ_0003416 expression levels in the plasma of mild, moderate, and severe PAH groups

3.2 | Validation of hsa_circ_0003416

According to the circBase database, hsa_circ_0003416 (chrX: 12995025–12995149) was identified to be derived from exon three of the *thymosin beta 4 X-linked (TMSB4X)* gene, which is 124-base pair (bp) long. Specific divergent primers of hsa_circ_0003416 were used for qRT-PCR, specificity being revealed by melting curve having a single peak (Figure 1A) and gel electrophoresis showing a single band of the expected size (Figure 1B). Agarose gel electrophoresis of the RT-PCR product revealed that divergent primers only amplified hsa_circ_0003416 in cDNA samples, whereas the convergent primers amplified the linear product in both cDNA

TABLE 4 Correlations of hsa_circ_0003416 with various parameters

Parameter	Correlation coefficient	*p*-Value
Age	0.136	0.178
Gender	−0.038	0.706
BMI	0.069	0.498
SBP	−0.027	0.790
DBP	−0.139	0.170
EF	−0.052	0.607
Cardiothoracic ratio	−0.135	0.181
sPAP	−0.177	0.079
mPAP	0.159	0.270
dPAP	−0.152	0.132
PVR	−0.161	0.132
Qp/Qs	−0.153	0.140
CI	0.180	0.081
BNP	−0.342	0.013
CK-MB	−0.167	0.098

Abbreviations: BMI, body mass index; BNP, B-type natriuretic peptide; CI, cardiac index; CK-MB, creatine kinase-MB; DBP, diastolic blood pressure; dPAP, diastolic pulmonary arterial pressure; EF, ejection fraction; mPAP, mean pulmonary arterial pressure; PVR, pulmonary vascular resistance; QP/QS, pulmonary to systemic flow ratio; SBP, systolic blood pressure; sPAP, systolic pulmonary arterial pressure.

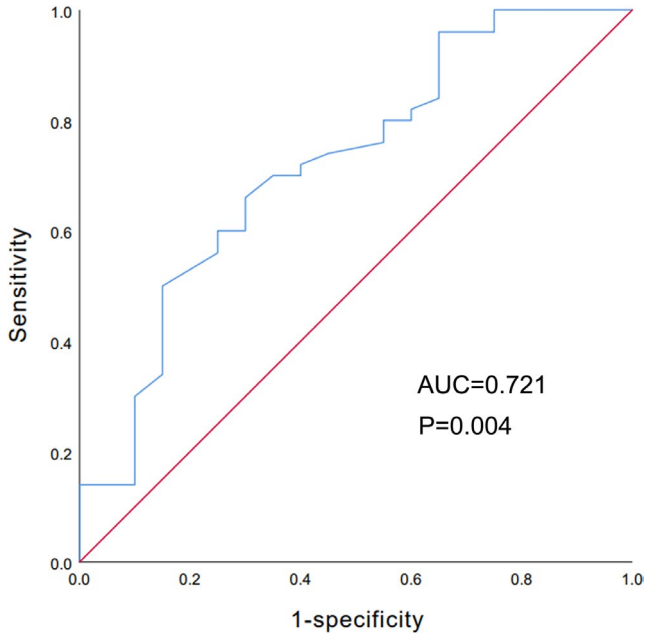

FIGURE 3 Receiver operating characteristic (ROC) curve of plasma hsa_circ_0003416

and gDNA (Figure 1C). Furthermore, the back-spliced junction of hsa_circ_0003416 was verified by Sanger sequencing (Figure 1D). The results collectively suggested that hsa_circ_0003416 was a circRNA.

3.3 | Expression of hsa_circ_0003416 in plasma of PAH-CHD patients

The hsa_circ_0003416 expression levels in plasma were determined in 50 PAH-CHD cases, 50 CHD cases, and 20 healthy cases by qRT-PCR, and the levels were found to be lower in the PAH-CHD group than in the CHD and healthy control groups ($p = 0.009$ vs. control group, $p = 0.026$ vs. CHD group). However, no significant difference was detected between the hsa_circ_0003416 levels of the healthy control and that of the CHD groups (Figure 2A). Furthermore, dividing PAH-CHD patients into three groups according to mPAP (mild: 20–40 mmHg, moderate: 41–55 mmHg, and severe: >55 mmHg) revealed that hsa_circ_0003416 expression levels had no significant difference across the three groups (Figure 2B). Additionally, the PAH-CHD patients were divided into different cardiac lesion groups (Table 3); there was no significant difference in hsa_circ_0003416 levels across the groups ($p > 0.05$).

3.4 | Spearman's correlation analysis of plasma hsa_circ_0003416 and clinical variables

The clinical variables, including SBP, DBP, EF, cardiothoracic ratio, sPAP, mPAP, dPAP, PVR, QP/QS, CI, BNP, and CK-MB, were significantly different between the PAH-CHD and CHD groups. Further analysis of their correlations with hsa_circ_0003416 expression levels showed hsa_circ_0003416 to be negatively correlated with BNP ($r = -0.342$, $p = 0.013$). However, there was no correlation between hsa_circ_0003416 and the other clinical characteristics (Table 4).

3.5 | Assessment of the diagnostic potential of hsa_circ_0003416 in patients with PAH-CHD

ROC analysis, for evaluating the potential diagnostic capability of plasma hsa_circ_0003416 levels for PAH-CHD, revealed that area under the curve (AUC) of plasma hsa_circ_0003416 was 0.721 (95% confidence interval = 0.585–0.857, $p = 0.004$), with a cutoff value of 0.99. Moreover, the sensitivity was 0.66 and specificity was 0.7 (Figure 3). Multivariate regression analysis was conducted to explore the predictive value of hsa_circ_0003416. The results indicated hsa_circ_0003416 (odds ratio [OR] = 0.015, 95% confidence interval = 0.000–0.597, $p = 0.025$) as an independent predictor of PAH-CHD (Table 5).

3.6 | Bioinformatics analysis

The circMIR software prediction revealed that hsa_circ_0003416 contained multiple miRNA-binding sites (Figure 4); the target genes of miRNAs were also predicted. The top 10 GO terms based on the three aspects (biological process [BP], cellular component

TABLE 5 Multivariate logistic regression analysis

Variable	B	SE	Wald	OR	95% CI	*p*-Value
hsa_circ_0003416	−4.199	1.879	4.993	0.015	0.000–0.597	0.025
Age	−0.328	0.167	3.833	0.721	0.519–1.000	0.050
Male	0.795	1.072	0.549	0.452	0.271–18.108	0.459
BMI	−0.495	0.316	2.457	0.609	0.609–0.328	0.117
BNP	0.000	0.000	0.252	1.000	1.000–1.000	0.616

Abbreviations: 95% CI, 95% confidence intervals; B, regression coefficient; BMI, body mass index; BNP, B-type natriuretic peptide; OR, odds ratio; SE, standard error.

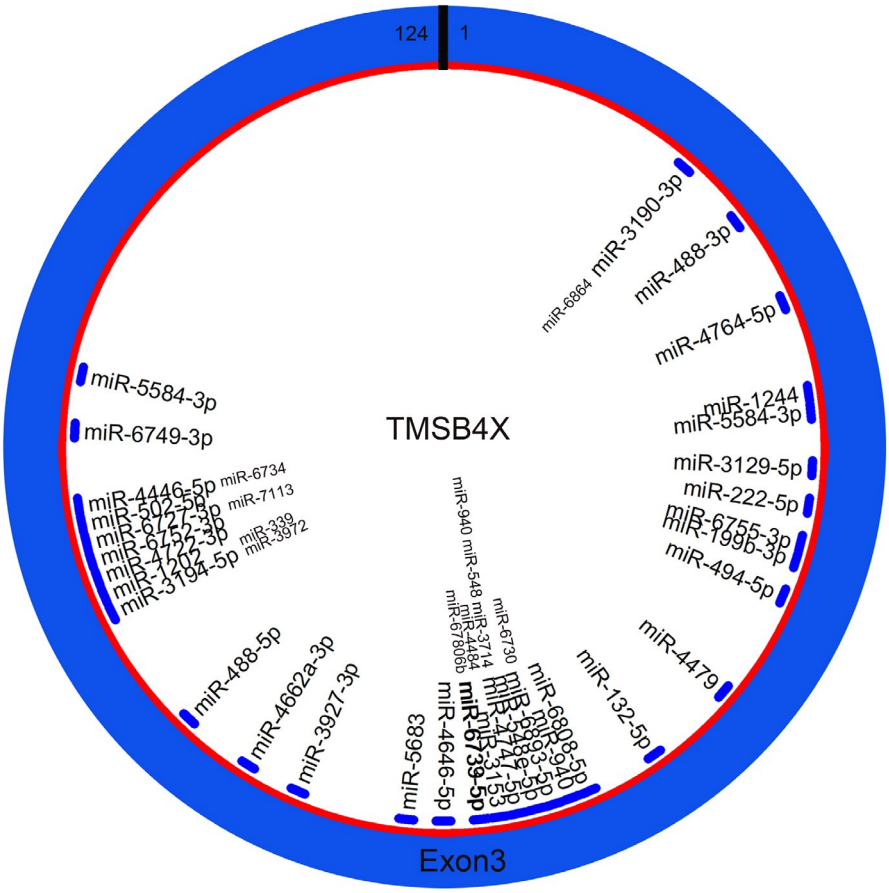

FIGURE 4 MicroRNA (miRNA)-binding sites of hsa_circ_0003416

[CC], and molecular function [MF]) are presented in Figure 5. KEGG pathway analysis indicated that the genes were primarily enriched in pathways associated with cancer. Notably, the forkhead box O signaling pathway was the most significantly enriched pathway. Among the top 11 pathways, the phosphatidylinositol-3-kinase and protein kinase B (PI3K-AKT) and transforming growth factor-β (TGF-β) signaling pathways have been proven to be involved in PAH (Figure 6).

4 | DISCUSSION

Pulmonary arterial hypertension, which is a common complication of CHD, increases the mortality risk of CHD patients.[26] Up to 30% of adult and 75% of pediatric PAH cases occur secondary to CHD.[27] CHD results in a systemic-to-pulmonary shunt that increases pulmonary blood flow, eventually leading to endothelial cell injury, neointimal development, and pulmonary vascular remodeling.[28] However, the development of PAH-CHD is a multistep and multifactorial process, and its pathogenesis has not yet been fully elucidated. In addition to hemodynamics-based changes, genetic and epigenetic alterations have been shown to contribute to the pathogenesis of PAH-CHD.[27,29] Accumulating evidence has revealed circRNAs to be implicated in the pathogenesis of PAH. For example, Zhou et al.[30] reported that hsa_circ_0016070 participates in the pathogenesis of PAH by promoting vascular remodeling via the miR-942/cyclin D1 axis. Miao et al.[31] found that hsa_circ_0046159 is correlated with chronic thromboembolic pulmonary hypertension. In addition, circ-calm4 has been shown to promote pulmonary vascular remodeling in hypoxic pulmonary hypertension.[24] However, there have been very few studies on the functional relevance of circRNAs in PAH-CHD.

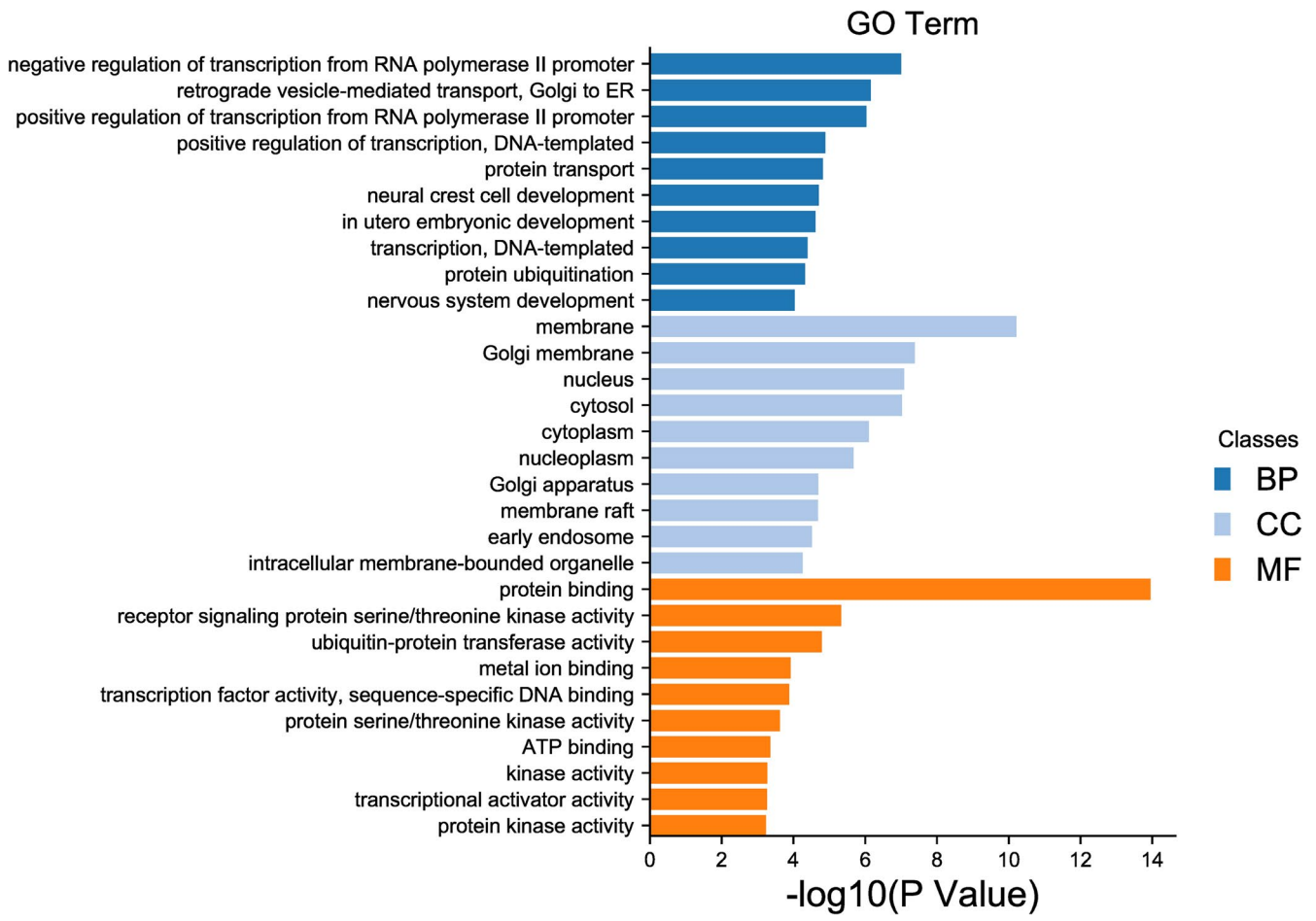

FIGURE 5 Gene ontology (GO) analysis of predicted target genes of hsa_circ_0003416

In the current study, the expression and clinical significance of hsa_circ_0003416 in PAH-CHD were explored for the first time, revealing that hsa_circ_0003416 may have potential regarding the diagnosis of PAH-CHD.

Currently, cardiac catheterization remains the gold standard for PAH diagnosis; however, it may introduce some risks for the patients due to its invasive nature. Although some non-invasive markers have been identified for the diagnosis and evaluation of PAH, there still remain some limitations in their clinical application. Recently, many studies have shown that circRNAs can serve as potential biomarkers for disease diagnosis. CircRNAs widely exist in eukaryotes at expression levels that are tenfold or higher than their linear isomers.[32] They have some crucial biological properties, such as specific expression, high conservation, high stability, and high abundance.[33] These characteristics indicate the promising potential of circRNAs as ideal biomarkers. Huang et al.[34] revealed that hsa_circ_0000745 levels are lower in the plasma and gastric cancer tissues of patients with gastric cancer than in control samples, indicating it as a promising diagnostic biomarker. Yuan et al.[35] reported that the circRNA circ_0026344 suppresses progression of colorectal cancer, and hence, can be utilized as a prognostic biomarker. Hang et al.[36] found circFARSA to be potent as a biomarker for lung cancer. These reports collectively suggested that circRNAs may open new possibilities for the early detection of diseases.

In recent years, several circRNAs have been identified as potential biomarkers for PAH. Zhang et al.[37] found circ_0068481 to be highly expressed in the serum of patients with idiopathic PAH (IPAH), with an AUC of 0.895 being obtained through ROC analysis. Its expression levels were able to predict a poor clinical outcome. This suggested that circ_0068481 could play the role of a serum biomarker of IPAH. Miao et al.[31] had reported that hsa_circ_0046159 expression is significantly increased in the blood samples of patients with chronic thromboembolic pulmonary hypertension. Another adult study reported the level of hsa_circ_0029642 to be significantly lower in PAH-CHD patients than in CHD patients, thereby suggesting hsa_circ_0029642 as a potential serum biomarker of PAH.[38] However, ours was the first study to explore the diagnostic role of circRNA in pediatric PAH-CHD. Here, hsa_circ_0003416 levels were found to be lower in PAH-CHD patients than in CHD patients and healthy subjects, although no significant difference was found across different PAH groups and CHD lesion groups. This lack of significance might relate to the relatively small sample size. Furthermore, hsa_circ_0003416 expression levels were found to be negatively correlated with BNP. BNP is secreted from ventricular myocytes

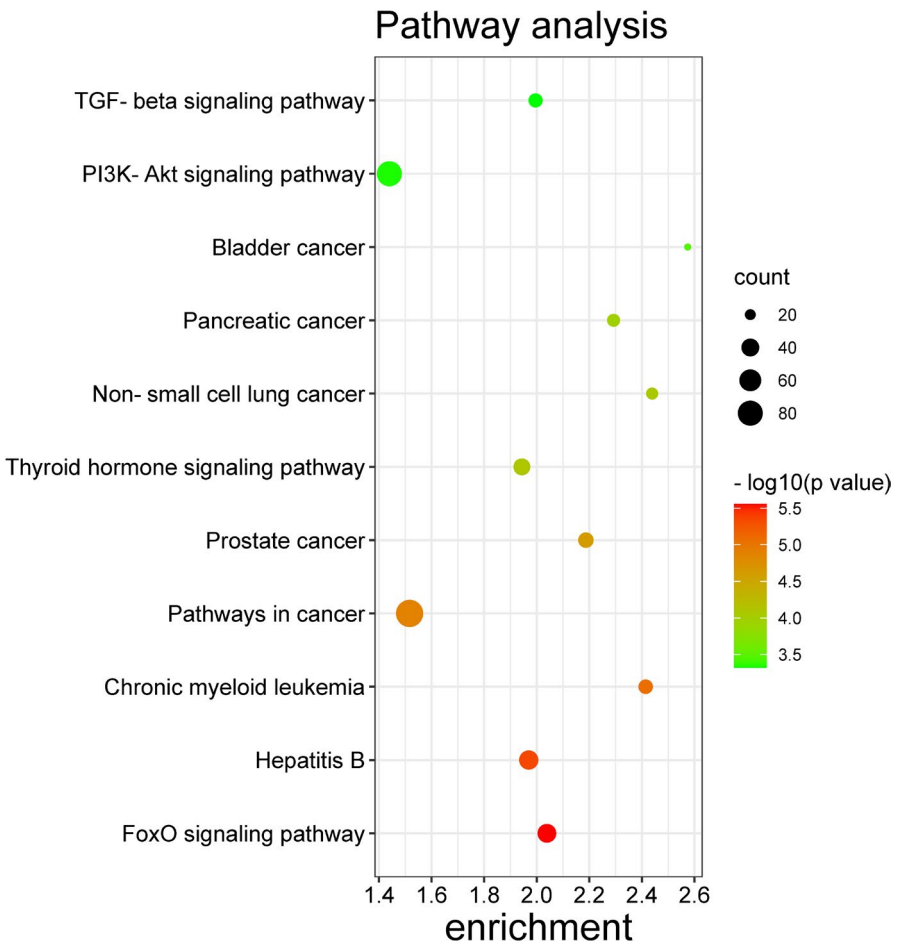

FIGURE 6 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of predicted target genes of hsa_circ_0003416

in response to pressure overload or hormonal stimulation. It is positively associated with mPAP and negatively associated with CI in patients; it can also be used for risk stratification in PAH.[39] Collectively, the results presented here suggested that plasma hsa_circ_0003416 may be related to the development and severity of PAH. Constructing ROC curves to further explore its diagnostic value revealed the AUC of plasma hsa_circ_0003416 to be 0.721, whereas the sensitivity was 0.66 and specificity was 0.7. Furthermore, hsa_circ_0003416 was considered to be an independent predictor of PAH-CHD. The results revealed that plasma hsa_circ_0003416 has potential as a diagnostic biomarker of PAH-CHD. However, exploring a new biomarker is a long and difficult process, from its discovery to validation and clinical application. Further validation would be required to evaluate the reliability of our current findings and the potential value of hsa_circ_0003416 in clinical application.

CircRNAs represent a relatively new field of research. To the best of our knowledge, there has been no definite evidence demonstrating the function of hsa_circ_0003416 till date. In this study, in silico analysis demonstrated the full-length hsa_circ_0003416 to be 124-bp long, encoded by the *TMSB4X* gene. *TMSB4X* is known as an important regulator of angiogenesis.[40] Numerous studies have indicated that circRNAs can act as miRNA sponges or competing endogenous RNA (ceRNA) and can regulate the downstream expression of genes.[16,41] A representative circRNA named Cdr1as has been reported to harbor over 70 miRNA-binding sites and regulate downstream pathways by inhibiting miR-7 activity.[16,42] Here, circRNA-miRNA interaction predictions revealed that hsa_circ_0003416 is able to interact with many miRNAs. GO analysis of target genes of the predicted miRNAs showed them to be primarily associated with terms, such as the regulation of transcription, retrograde vesicle-mediated transport, and protein binding. Moreover, KEGG analysis suggested that most of the predicted genes were primarily enriched in pathways associated with cancer. As is well known, PAH and cancer share similar phenotypes, such as hyperproliferation, over-migration, and anti-apoptosis. Furthermore, among the top 11 pathways, the PI3K-AKT and TGF-β signal pathways have been proven to be involved in PAH.[43,44] The results presented here revealed that hsa_circ_0003416 may contribute to the pathogenesis of PAH through a ceRNA mechanism. However, mechanism underlying the aberrant expression of hsa_circ_0003416 in PAH has not yet been elucidated; therefore, further research would be required for its validation.

The present study had some limitations. First, the sample size was relatively small; a larger sample size would be required in future studies for validating the results. Second, the expression level of hsa_circ_0003416 should be investigated before and after surgery to assess the correlation between hsa_circ_0003416 and clinical data. Third, the underlying mechanism by which

hsa_circ_0003416 affects PAH-CHD pathogenesis was not elucidated; thus, further experiments would be required to determine the exact mechanism.

5 | CONCLUSION

This study determined, for the first time, the expression levels of hsa_circ_0003416 in plasma of PAH-CHD patients, CHD patients, and healthy subjects in a pediatric population. The obtained results indicated that hsa_circ_0003416 might serve as a candidate biomarker for diagnosing PAH-CHD. The findings of this study provided new insights into the diagnosis of PAH-CHD.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

YH conducted data analysis and drafted the manuscript. YH and DS performed the experiments. BY, YH, SQ, CC, and YZ collected the specimens and clinical information. YP made contribution to study design and revised the manuscript. All authors agreed to publication of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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