Original Article

Bioinformatic analysis of dysregulated circular RNAs in pediatric pulmonary hypertension linked congenital heart disease

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Background: Circular RNAs (circRNAs) may play important roles in the progression of pulmonary arterial hypertension. However, the potential roles they play in childhood pulmonary arterial hypertension associated congenital heart disease (CHD) progression remains unclear.

Methods: Thirteen human plasma samples including eight from pulmonary arterial hypertension secondary to CHD patients and five from a control group were analyzed using the Arraystar Human circRNA array. The relative expression levels of five differentially expressed circRNAs in pulmonary arterial hypertension were detected using real-time polymerase chain reaction (PCR) analysis. In parallel, these levels were also taken on control samples from 32 CHD patients. We used miRanda and TargetScan software packages to predict potential microRNA (miRNA)targets, which were then combined into a circRNA–miRNA–messenger RNA (mRNA) network.

Results: Twenty-seven circRNAs (three upregulated and 24 downregulated) were differentially expressed between the pulmonary arterial hypertension and control groups. Compared to control group levels, circ_003416 expression in the pulmonary arterial hypertension group was significantly downregulated, while circ_005372 expression, in contrast, was significantly upregulated. The differential expression of these circRNAs was mainly linked to variation in levels of oxidative phosphorylation and tight junction signaling.

Conclusions: We identified one overexpressed and one underexpressed circRNA in plasma samples from children with CHD associated pulmonary arterial hypertension. Bioinformatic analysis indicated these dysregulated circRNAs might be associated with the occurrence and regulation of pulmonary arterial hypertension.

Keywords: Circular RNA (circRNA); pulmonary hypertension; congenital heart disease (CHD); bioinformatic analysis; children

Submitted Mar 11, 2022. Accepted for publication Apr 28, 2022.
doi: 10.21037/tp-22-117
View this article at: https://dx.doi.org/10.21037/tp-22-117

Introduction

Pulmonary artery hypertension (PAH) is a term encompassing a cluster of diseases characterized by increased pulmonary vascular resistance. It is a particularly prevalent and critical complication of left-to-right shunt congenital heart disease (CHD), a condition which can lead to right ventricular failure and even sudden death. CHD is the most important cardiovascular disease and the most common birth defect in children. According to surveys conducted in many regions of China over the past

decade, the incidence of CHD is approximately 4–22%, indicating it places a serious burden on families and society (1,2). Compared to adults, a low incidence of idiopathic pulmonary arterial hypertension (IPAH) is found in children. Further, approximately 24–52% of pediatric PAH cases are associated with CHD (CHD-PAH), reflecting a higher incidence amongst CHD groups (3,4). Even after complete surgical CHD repair, PAH persistent or recurrent children experience significant pulmonary vascular changes like those with IPAH (5). While its pathogenesis has not yet been fully elucidated, data have shown key genetic risk factors associated with CHD-PAH can lead to a lack of PAH treatment response (6). This leads to rapid disease progression, even when the hemodynamic manifestations are mild or similar. However, the underlying etiology of this phenomenon remains unclear (7,8).

Therefore, in addition to hemodynamic manifestations, it is crucial to study PAH etiology and pathogenesis, as this would aid to reverse pulmonary vascular disease and explore new targets for preventing and treating the condition. While most mammalian genomes are transcribed into RNA, only a small number of said transcripts are translated into proteins. An increasing number of noncoding RNAs have been found that do not encode homologous peptides, yet they still play a major role in the regulation of many basic cellular processes and tissue-specific physiological functions (9). Circular RNAs (circRNAs) comprise a sub-category of these functionally vital, yet noncoding RNAs and are characterized by a covalently closed loop structure, and very stable and not easily degraded expression. CircRNAs share distinct advantages in the development and application of effective treatments for cardiovascular diseases, especially in fields centered on developing novel clinical diagnostic markers and molecular targeted therapies (10,11). In humans, mice, and nematodes, previous reports have shown circRNAs act as microRNA (miRNA) sponges (12), and also participate in the transcriptional regulation of cancer, neurological diseases, and other pathologies (13). PAH and cancer cells show similar high proliferation and anti-apoptotic phenotypes, and studies have confirmed PAH links to differentially expressed circRNAs (14,15). However, the biological functions and regulatory mechanisms of PAH circRNAs remain elusive, and questions surrounding their role in the condition present imperative scientific problems worthy of in-depth research. We applied microarray analysis to identify circRNAs differentially expressed between PAH patients and CHD control subjects, then verified their expression levels in a larger sample. Prediction of miRNAs regulated by circRNAs and their downstream target genes were then developed. Using bioinformatic methods, we can now construct circRNA-related targeted regulatory pathways to attain further insight. We present the following article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-117/rc).

Methods

Patients and samples

The key aspects of study design (including the research question, primary outcome to be measured and the statistical analysis plan) were prepared and registered before data collection began. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University [No. 2021(KY-E-050)], and parental consent was sought and obtained prior to all research. Eight patients with CHD-PAH (PAH group) and five with PAH-free CHD (the control group) were enrolled in the microarray study, while 16 patients from each group (PAH and control) were enrolled in our validation study. All patients were hospitalized at the Department of Pediatrics from May 2020 to December 2020. PAH was diagnosed by right-heart catheterization based on criteria set as a pre-intervention, with a mean resting pulmonary arterial pressure of ≥25 mmHg. Patients who were diagnosed with other systemic diseases, such as pulmonary dysplasia, arrhythmia, or associated disease, were excluded, as were those who received targeted therapy for PAH. The characteristics of the 13 patients who provided samples for the microarray study are summarized in *Table 1*. In our validation study, 7 boys and 9 girls were included in the CHD group, with a median age of 4 (3.48–5.38) years, while 9 boys and 7 girls were included in the PAH group, with a median age of 2.75 (0.8–5.48) years.

Blood was collected from the femoral vein before cardiac catheterization, and the plasma was separated by centrifugation at 3,000 rpm for 15 min then held at –80 ℃ until total RNA was isolated.

circRNA expression-profile data

The purity and concentration of total RNA was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific,

Table 1 Baseline characteristics of 13 patients who provided samples for analysis

Patient ID	Group	Gender	Weight (kg)	Age (y)	Diagnosis	CHD diameter (mm)	PAP [mmHg]
HYK	PAH	M	9.2	1.3	VSD	8.8	66/34 [40]
HY	PAH	M	16.8	9.2	ASD	39	86/53 [64]
LHJ	PAH	F	11.0	4.2	VSD	18	85/44 [58]
MJQ	PAH	F	16.4	5.8	VSD	18	73/44 [54]
TWM	PAH	M	5.6	0.4	VSD	14	66/30 [41]
PBC	PAH	M	6.3	0.58	VSD	9	68/25 [42]
WLY	PAH	F	5.2	0.8	VSD	10	90/48 [63]
HXH	PAH	M	8.3	1.8	*	NA	85/42 [56]
TBQ	Control	M	32.4	12.8	VSD	12	27/14 [18]
WYY	Control	M	12.4	3.9	VSD	9	22/10 [14]
TBY	Control	F	13.8	3.8	VSD	5	25/10 [15]
ZQY	Control	F	13.2	3.5	VSD	5.1	21/10 [14]
TYX	Control	M	14.5	3.0	VSD	5	17/11 [14]
P value		1.0	0.06	0.26	1.0	NA	<0.01

*, ectopic origin of right pulmonary artery from the ascending aorta and PDA. CHD, congenital heart disease; PAP, pulmonary artery pressure; PAH, pulmonary artery hypertension; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus.

USA). After digestion with RNase R (Epicentre, Inc., USA), the enriched circRNAs were then amplified and transcribed into fluorescent complementary RNA (cRNA) utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar, USA). The labeled cRNAs were hybridized to the Human circRNA Array (8×15 K, Arraystar) and array pictures were then scanned and analyzed using the Agilent software package. R software was used for data analysis and subsequent processing. Differential circRNA expression between the two samples was identified by filtering and supported fold-changes, and distinguishable circRNA expression patterns within the samples were identified by hierarchical clustering. The volcano plot shows known differentially expressed circRNAs. Student's *t*-tests were used to calculate statistical significance, while a fold-change of >1.5 with a P value of <0.05 served as our cutoff criterion for significant differential expression.

Reverse transcription and quantitative polymerase chain reaction ((RT-qPCR) validation

Total RNA, including circRNAs, was extracted from the plasma using a BIOG cfRNA Easy Kit (Changzhou Baidai Biotechnology Co., Ltd., China) according to the manufacturer's instructions. Plasma total RNA was reverse transcribed into complementary DNA (cDNA) using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd., China), and subsequent real-time PCR assays were then performed with plasma samples from PAH patients and CHD controls. All primers were designed and synthesized by Guangzhou Geneseed Biotech (*Table 2*). The qPCR results were normalized to the reference gene GAPDH. circRNA relative expression levels were then calculated using the $2^{-\Delta\Delta Ct}$ method (16).

Functional analysis and regulative network

circRNA–miRNA and miRNA–messenger RNA (mRNA) interactions were predicted using Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda (17,18). We then studied the miRNA-response elements (MREs) related to qRT-PCR differentially expressed circRNAs to explore the circRNA–miRNA interactions in detail. Target miRNA genes were predicted using Arraystar's software, then Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to predict the functions of hsa_circ_005372 and hsa_circ_003416. A circRNA–

Table 2 Sequences of primers used to amplify circRNAs

Name	Reverse (5′–3′)	Forward (5′–3′)
hsa_circ_005372	TTAAACTGCGGATTGCTTGT	CTCTCATCAAGCCTGCATCA
hsa_circ_101465	TCAGGTTCAGGAGCACGGGAAT	TCATGTATGCCCTGGCCTTCGGT
hsa_circ_010921	GCCTGCCAACCTCACCATTC	GCAGATGGGAAAGCGATGGC
hsa_circ_003416	ATTTAAACTTGATCCAACATGC	CCCCTTTCACATCAAAGAAC
hsa_circ_008882	TAATGCTAGGCTGCCAATGGT	GCAGGAATACCTTTCCTCACAG

circRNA, circular RNA.

miRNA–mRNA regulatory network was then established using Cytoscape (http://www.cytoscape.org/).

Statistical analysis

All statistical analyses were performed using SPSS version 25.0 (SPSS, USA). Intergroup clinical or demographic variation was determined by either Student's *t*-test or Fisher's exact test, and Mann-Whitney U tests were performed to determine the relative circRNA expression levels. Statistical significance was set at P<0.05, and every applied mathematics test was bilateral.

Results

circRNA profiles in PAH and control samples

In our systematic study of PAH circRNA expression levels, we performed circRNA microarray analysis of both PAH and control groups. Hierarchical cluster analysis showed the circRNA expression patterns were very different between these groups, as reflected in red and green shading points for high and low respective circRNA expression levels seen in *Figure 1A* (fold change >1.5, P<0.05). Red dots in the volcano map represent circRNAs that behaved markedly differently between the two samples, with a fold change of >1.5 (*Figure 1B*). The overall results suggest that 11,611 circRNAs were differentially expressed in PAH samples (fold change ≥1), with three circRNAs significantly upregulated and 24 circRNAs significantly downregulated (fold change ≥1.5, P<0.05).

Validation of dysregulated circRNAs

We selected five dysregulated circRNAs to further validate our microarray expression results, including two upregulated circRNAs (005372, 101465) and three downregulated circRNAs (010921, 003416, 008882). To achieve this, we selected based on the following criteria: raw intensity >100, fold change ≥1.5, P<0.05. Samples from 32 patients with CHD, including 16 with CHD-PAH, were included in the validation group, and qRT-PCR was performed to detect the differential expression levels in the patient's plasma samples. In agreement with the microarray results, hsa_circ_005372 was significantly dysregulated between the case and control groups (*Figure 2A*). By contrast, hsa_circ_101465, hsa_circ_010921, and did not differ significantly between the two groups (*Figure 2B*,*2C*). Hsa_circ_003416 was significantly dysregulated (*Figure 2D*), while hsa_circ_008882 did not differ significantly between the two groups (*Figure 2E*).

Prediction of miRNA binding to differentially expressed circRNAs

The miRNAs predicted to be associated with twenty-seven differentially expressed circRNAs are summarized in *Table 3*. Among them, hsa_circ_407339 and hsa_circ_405324 were associated with the highest number of miRNAs (1,863 and 939, respectively).

MREs that could describe miRNA binding to hsa_circ_005372 and hsa_circ_003416 were also predicted, and the top five MREs predicted for hsa_circ_005372 were miR-548g-3p, miR-365b-5p, miR-365a-5p, miR-216a-5p, and miR-873-5p (*Figure 3A*). The top five MREs predicted for hsa_circ_003416 were miR-1244, miR-4747-5p, miR-6755-3p, miR-548an, and miR-3194-5p (*Figure 3B*).

Functional analysis of qRT-PCR-verified deregulated circRNAs

GO function and KEGG enrichment analyses were performed for the two circRNAs showing significant

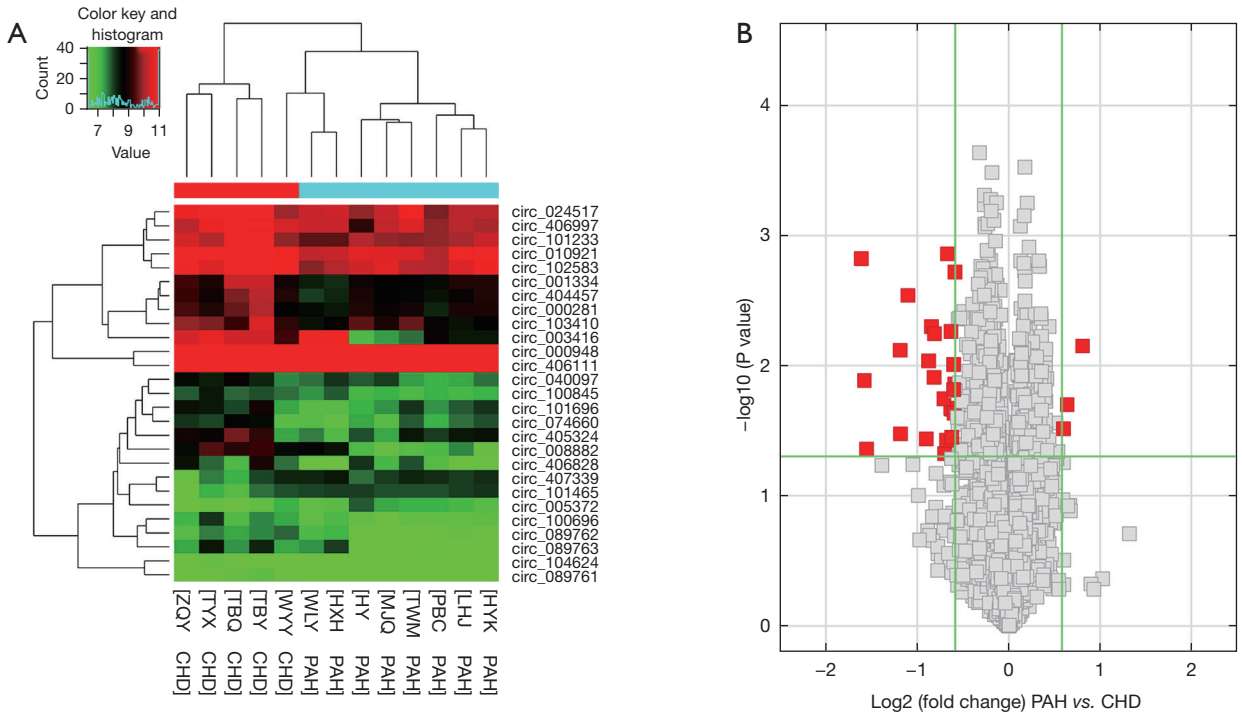

Figure 1 Plasma circRNAs expression profiles in PAH and control samples. (A) Hierarchical-cluster map. The red and green shading respectively represents high and low expression levels. (B) Volcano map. The X-axis is denoted by $\log_2$ (fold change) and Y-axis is denoted by $-\log_{10}$ (P value). The green vertical lines represent a 1.5-fold change upregulation or downregulation and the green horizontal line represents a P value of 0.05. Differential expression of circRNAs that were statistically significant are represented by red squares in the plot. circRNA, circular RNA; PAH, pulmonary artery hypertension; CHD, congenital heart disease.

differential expression by qRT-PCR, and we identified 51 GO biological processes (BPs), 10 GO cellular components (CCs), 10 GO molecular functions (MFs), 71 GO functions, and seven KEGG pathways. The differentially expressed circRNAs were related to terms such as cellular response to retinoic acid, cellular protein complex disassembly, platelet alpha granule, cyclin-dependent protein kinase holoenzyme complex, endopeptidase inhibitor activity, and enzyme inhibitor activity (*Table 4*). Oxidative phosphorylation and tight junction signaling pathways proved the most enriched pathways associated with PAH (*Table 5*).

Construction of a circRNA–miRNA–mRNA regulative network

Having predicted MREs and targeted genes, and as informed by qRT-PCR results confirming very different circRNA expression levels, we built a regulative interaction map of PAH-related circRNA–miRNA–mRNA. This schematic included 93 mRNAs and 29 miRNAs for hsa_circ_005372, and 26 mRNAs and 19 miRNAs for hsa_circ_003416 (*Figure 4*).

Discussion

Vascular endothelial functionality disorders link to a chronic state of high pulmonary blood flow. In turn, this leads to imbalances in various vascular contraction and diastolic factors between collagen generation and degradation, causing pulmonary vascular remodeling which eventually leads to PAH in left-to-right shunt CHD patients (19). Nonetheless, a PAH pathogenic mechanism has yet to be completely elucidated. Other studies have shown that the occurrence of CHD in PAH patients cannot be completely explained by hemodynamics, genetic mutations, or clinical phenotype related epigenetic modification (20-22), and mounting evidence has indicated that wide ranges of circRNAs are abnormally expressed in PAH (23-25). However, the specific involvement of circRNAs in CHD-PAH pathogenesis merits further study. For the first time,

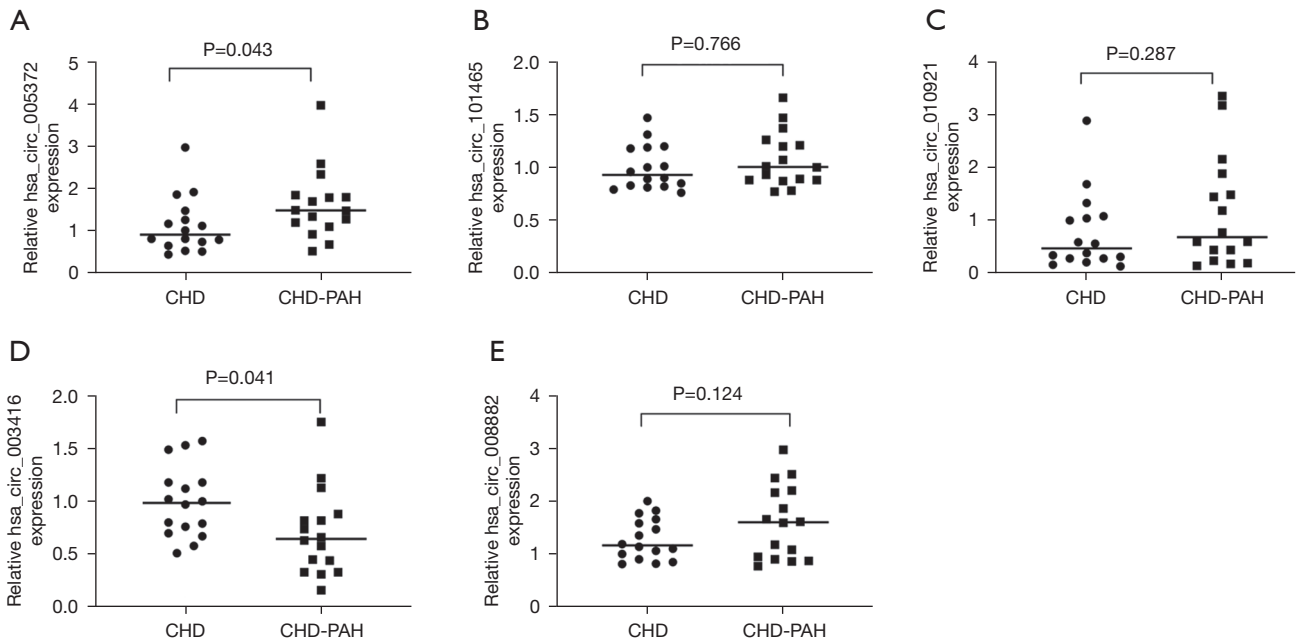

Figure 2 Relative expression levels of five differentially expressed circRNAs. (A) hsa_circ_005372 gene. (B) hsa_circ_101465 gene. (C) hsa_circ_010921 gene. (D) hsa_circ_003416 gene. (E) hsa_circ_008882 gene. The horizontal bars in the scatter chart represent the median expression values. circRNA, circular RNA, CHD, congenital heart disease; PAH, pulmonary artery hypertension.

this study analyzed plasma circRNA expression levels in children with CHD-PAH, and identified numerous circRNAs that were aberrantly expressed compared with children with PAH-free CHD.

We identified and analyzed circRNA plasma levels in PAH patients with CHD to further our efforts to explore their potential pathogenic roles. As a result, we found certain circRNAs were differentially expressed in PAH, and two were the most significantly dysregulated in children with the condition. Application of GO and KEGG analyses then revealed BPs and signaling pathways linked to both of these differentially expressed circRNAs. Bioinformatic analysis indicated that both might be related to GO phenomena, including endopeptidase inhibitor activity, enzyme inhibitor activity, platelet alpha granule, cyclin-dependent protein kinase holoenzyme complex, cellular response to retinoic acid, and cellular protein complex disassembly, while KEGG analysis suggested the oxidative phosphorylation and tight junction signaling pathways were significantly enriched. This is likely related to PAH occurrence and regulation processes as mentioned in previous studies (26,27). Abnormal regulation of oxidative signaling is closely related to cell apoptosis, and antioxidant therapy has been shown to have a certain effect on PAH (28). Changes in tight junction signaling may affect cell proliferation and migration (29), while the proliferation, migration, and apoptosis of pulmonary smooth muscle cells (PASMCs) are the main pulmonary vascular remodeling changes found in PAH (30). Therefore, these two abnormally regulated circRNAs and their related signaling pathways are expected to become new therapeutic targets for PAH, although the mechanics informing their regulation of PASMC proliferation, migration, and apoptosis merits further study.

In recent years, the mechanisms of different PAH circRNAs have been reported. In a previous study, serum hsa_circ_0029642 expression was significantly lower in patients with CHD-PAH compared to patients with normal pulmonary artery pressure, suggesting it could serve as a new serum marker for PAH (31), and similar results have been observed in other types of PAH. Wang *et al.* (24) performed circRNA microarray and bioinformatic analyses on lung tissues from hypoxic pulmonary hypertensive mice and found several potentially diagnostic and therapeutic circRNA targets for the condition. Further experimentation revealed circRNAs are related to vascular remodeling and PAH pathogenesis by affecting the proliferation of miRNA promoted PASMCs (32,33). In another study, Miao *et al.* (14) analyzed the circRNA profile and identified hsa_

Table 3 Predicted numbers of target miRNAs associated with differentially expressed circRNAs

Name	Regulation	Number of miRNA targets
hsa_circ_407339	Up	1863
hsa_circ_405324	Down	939
hsa_circ_001334	Down	903
hsa_circ_000948	Down	650
hsa_circ_406111	Down	386
hsa_circ_406997	Down	346
hsa_circ_089763	Down	222
hsa_circ_074660	Down	220
hsa_circ_089761	Down	192
hsa_circ_404457	Down	146
hsa_circ_089762	Down	108
hsa_circ_406828	Down	97
hsa_circ_040097	Down	79
hsa_circ_005372	Up	78
hsa_circ_000281	Down	78
hsa_circ_010921	Down	53
hsa_circ_003416	Down	45
hsa_circ_101233	Down	43
hsa_circ_104624	Down	42
hsa_circ_102583	Down	41
hsa_circ_101696	Down	33
hsa_circ_008882	Down	31
hsa_circ_100696	Down	31
hsa_circ_024517	Down	30
hsa_circ_100845	Down	23
hsa_circ_101465	Up	10
hsa_circ_103410	Down	8

miRNA, microRNA.

circ_0002062 and hsa_circ_0022342 as potential therapeutic targets in the peripheral blood of chronic thromboembolic pulmonary hypertensive patients. Similarly, Zhang (34) found serum circ_0068481 levels were elevated in patients with IPAH, suggesting it could be used to diagnose the condition, predict adverse clinical outcomes, and serve as a novel and non-invasive biomarker. Although the etiologies are different, with manifestations such as hypoxia and thromboembolism, circRNAs play important roles in the various types of PAH. The common molecular mechanisms that are evident in PAH are also reflected in its genetic etiology. In turn, identically variant gene expression was found across patients with CHD-PAH, connective tissue disease PAH, and IPAH (35). Further, in our present study, hsa_circ_005372 and hsa_circ_003416 were significantly dysregulated and may play a role in regulating the expression of both host genes and downstream target genes. However, questions as to whether these factors can be used as potential diagnostic biomarkers or therapeutic targets still merit further testing on larger samples.

The circRNAs, similar to long non-coding RNAs and mRNAs, contain many miRNA-binding sites and function through a variety of mechanisms. These mechanisms include acting as competitive endogenous RNAs (ceRNAs), regulating gene transcription, directly interacting with proteins, and influencing protein translation (36). The miRNA sponge-like functions of circRNAs, as well as their ability to regulate downstream miRNA-target gene expression as ceRNAs, comprise the most widely studied of these mechanisms (37). In one study, hsa_circ_0016070 was shown to be related to vascular remodeling and to participate in PAH pathogenesis by influencing miR-942/*CCND1* to promote pulmonary artery smooth muscle cell proliferation (38). In 2020, Miao *et al.* (39) predicted that, based on an analysis of peripheral blood samples, the hsa_circ_0046159–miR-1226-3p–*ATP2A2* pathway was linked to CTEPH progression.

To discern the specific biological consequences of the two abnormally expressed circRNAs, a network including circRNA–miRNA–mRNA interaction was constructed and revealed possible connections between circRNAs and their target genes. In addition, the network served as an important study reference on the regulatory mechanisms between circRNAs and their potential downstream targets. We observed that hsa_circ_005372 and hsa_circ_003416 could bind to many miRNAs, some of which have been associated with cell proliferation, migration, and differentiation. For example, miR-3125 (40), miR-216a-5p (41), and miR-365a-5p (42) were identified as a novel upregulated circRNA's targets (hsa_circ_005372) while, by contrast, miR-199a/b-3p, miR-3194-5p (43), and miR-486-3p (44) were identified as targets of a novel downregulated form of circRNA (hsa_ circ_003416). Among these, miR-199a-3p was previously linked to PAH pathogenesis, which was indicated by the prediction of potential hub

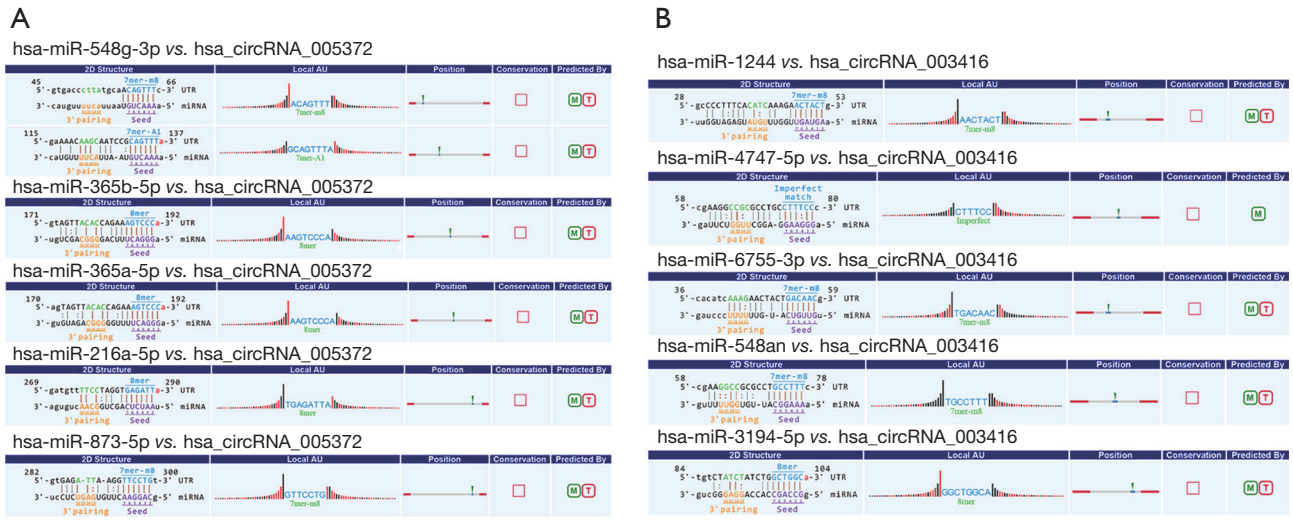

Figure 3 Detailed interaction information between hsa_circRNA_005372 (A), hsa_circRNA_003416 (B), and their matching miRNAs. Two-dimensional structures are shown for the MRE sequence, pairing with target-miRNA nucleotides 13–16, and the target miRNA seed type (8mer, 7mer-m8 or imperfect). The exact nucleotide positions are shown in the alignments in the upper left and right corners. The "local AU" refers to the 30 nucleotides downstream and upstream of the seed sequence. A/U and G/C was stand for red and black bars, which represent seed regions with high and low accessibility, respectively. The extent of accessibility was represented by height of each bar. The position column displays the closer to the sides, the better. circRNA, circular RNA; MRE, miRNA-response element.

gene targeting drugs (45,46), lending new insights into future therapies. Potential mRNA targets for miR-199a-3p include *TMSB4X* (47) and *ACTA2* (48), which are related to vasoconstriction and cell apoptosis and play key roles in PAH. While circRNAs and miRNAs may target many genes, resulting in changes in many downstream targets, a comprehensive understanding of their exact biogenesis and regulatory circuits is lacking at present (49). Research on the roles of circRNAs in the occurrence and regulation of PAHs is in its infancy, and further exploration is needed and anticipated.

The detection of circRNAs related markers is expected to provide a noninvasive detection method for the diagnosis, prognosis and treatment of PAH. However, the research of circRNAs in PAH is still in its infancy. There are still many challenges: first, the expression level of most circRNAs is low, and more advanced and sensitive technologies and tools need to be developed to detect the quantification and verification of circRNAs; Second, the sequence of most circRNA is synchronized with the mRNA produced by the host gene, so it also faces technical challenges, such as overexpression and silencing strategies (12). At the same time, the mechanisms of circRNA biogenesis are still fairly elusive. Current studies suggest that the biogenesis of circRNA is regulated by specific cis-acting elements and trans-acting factors (50,51). And a number of RNA-binding proteins regulate and control circRNA biogenesis. Gene body methylation also plays a regulatory role in circRNA biogenesis. In addition, epigenetic changes within histones and gene bodies affect alternative splicing and may also have a direct impact on circRNAs biogenesis. Exploring the factors that affect biogenesis will help us understand their function better. The biogenesis and exact function of circRNA and its function and mechanism in different human diseases are still unclear. Therefore, further research is needed in the future to make circRNA play a greater role in the prevention, diagnosis and treatment of PAH and realize clinical transformation faster.

One limitation of this study is that we only verified the differential expression of circRNAs in 16 pairs of patients, and a larger sample size is needed to confirm these results and long-term follow-up and further evaluation of circRNAs expression changes after treatment are required to develop biomarker circRNAs. Moreover, the specific function of these abnormally expressed PAH circRNAs and the degree to which they are involved in PAH pathogenesis still awaits cellular and animal model confirmation. To date, limited research has been conducted on the interactions between PAH circRNAs and miRNAs, and the results of our study have established a circRNA–miRNA–gene PAH

Table 4 Top five GO functions of circRNAs-target genes

GO.ID	Term	P value	Genes
Biological process			
GO: 0071300	Cellular response to retinoic acid	0.005	*BRINP3/NDUFA13/SERPINF1*
GO: 0043624	Cellular protein complex disassembly	0.006	*KIF2A/SYNJ1/VILL/MRPL16/MRPL40*
GO: 0010447	Response to acidic ph	0.007	*GIP/SERPINF1*
GO: 0070830	Bicellular tight junction assembly	0.007	*CLDN20/PARD6A/CRB3*
GO: 0120192	Tight junction assembly	0.008	*CLDN20/PARD6A/CRB3*
Cellular component			
GO: 0031091	Platelet alpha granule	0.012	*APLP2/TMSB4X/STXBP3*
GO: 0000307	Cyclin-dependent protein kinase holoenzyme complex	0.022	*CCNH/CCNB3*
GO: 0005923	Bicellular tight junction	0.028	*CLDN20/PARD6A/CRB3*
GO: 0005856	Cytoskeleton	0.032	*KIF2A/EIF6/CETN3/SPATC1/PARD6A/CCDC28B/CCDC15/CCNB3/ACTA2/TBCB/SYNJ1/KRTAP131/CCDC39/CTNNA2/VILL/ZBTB49/ODF3L1/RAI14/TMSB4X*
GO: 0070160	Tight junction	0.032	*CLDN20/PARD6A/CRB3*
Molecular function			
GO: 0004866	Endopeptidase inhibitor activity	0.017	*APLP2/SERPINF1/CST8/SMR3A*
GO: 0004857	Enzyme inhibitor activity	0.017	*PDC/ENSA/SMR3A/APLP2/SERPINF1/CST8*
GO: 0030414	Peptidase inhibitor activity	0.019	*SMR3A/APLP2/SERPINF1/CST8*
GO: 0061135	Endopeptidase regulator activity	0.020	*SMR3A/APLP2/SERPINF1/CST8*
GO: 0015026	Coreceptor activity	0.027	*LRP5/LILRA4*

GO, Gene Ontology, circRNA, circular RNA.

Table 5 KEGG analysis of two differentially expressed circRNAs

Pathway ID	Term	P value	Genes
hsa04662	B cell receptor signaling pathway	0.006	*IFITM1/LILRA1/LILRA4*
hsa03420	Nucleotide excision repair	0.020	*CCNH/LIG1*
hsa00190	Oxidative phosphorylation	0.023	*ATP6V1H/COX6A1/NDUFA13*
hsa05226	Gastric cancer	0.031	*CTNNA2/FGF3/LRP5*
hsa05131	Shigellosis	0.038	*DOCK1/U2AF1L4*
hsa04530	Tight junction	0.043	*CLDN20/CRB3/PARD6A*
hsa05100	Bacterial invasion of epithelial cells	0.045	*CTNNA2/DOCK1*

KEGG, Kyoto Encyclopedia of Genes and Genomes; circRNA, circular RNA.

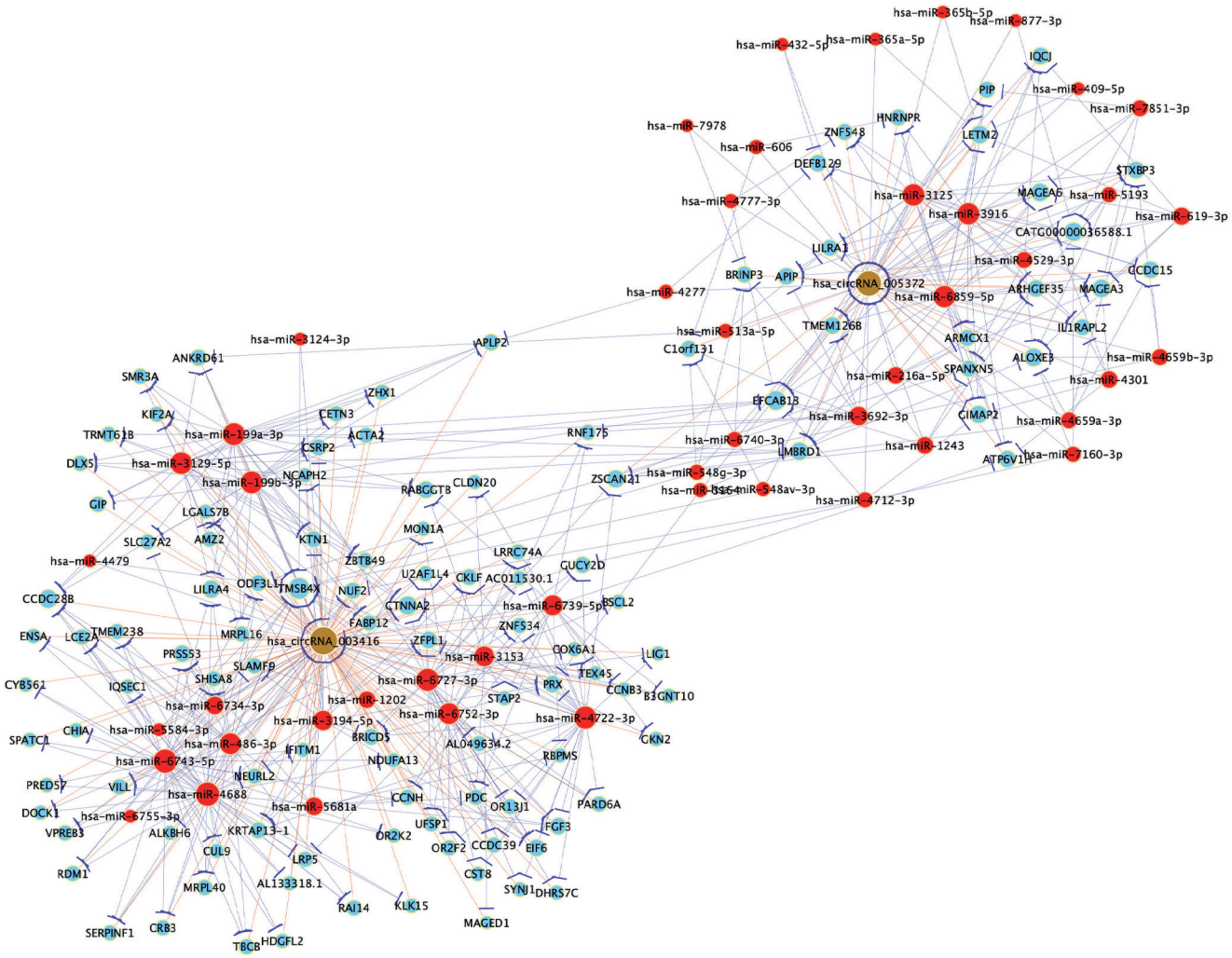

Figure 4 circRNA–miRNA–mRNA construction network. The regulative network contained hsa_circ_005372 and hsa_circ_003416, along with 48 downstream miRNAs and target mRNAs. Brown, red, and light-blue nodes respectively represent are circRNAs, miRNAs, and protein-coding RNAs, and an edge with a T-shaped arrow indicates a directed relationship. Edges without arrows represent undirected relationships (ceRNA relationships). circRNA, circular RNA; miRNA, microRNA; mRNA, messenger RNA; ceRNA, competitive endogenous RNA.

network which will inform further explorations of the roles circRNAs play in PAH pathogenesis.

In conclusion, we identified differentially expressed PAH circRNAs and showed their dysregulation may link to PAH pathogenesis. The results of this study can broaden perspectives on PAH epigenetic research and lay a preliminary foundation for future studies on the roles of PAH circRNAs.

Acknowledgments

The authors thank all participating families and patients for their contributions. The authors would also like to thank all physicians at The First Affiliated Hospital of Guangxi Medical University, Department of Pediatrics-Division I, for their help and support.

Funding: None.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-117/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-22-117/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-117/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to its accuracy and integrity are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University [No. 2021(KY-E-050)]. Written informed consent was obtained from the parents of all individual participants.

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Cite this article as: Su D, Huang Y, Liu D, Huang Y, Ye B, Qin S, Chen C, Pang Y. Bioinformatic analysis of dysregulated circular RNAs in pediatric pulmonary hypertension linked congenital heart disease. Transl Pediatr 2022;11(5):715-727. doi: 10.21037/tp-22-117