Plasma LPA protein levels are associated with postoperative persistent pulmonary hypertension in children with ventricular septal defects

Di Yu
Children's Hospital of Nanjing Medical University
Xiaodong Zang
Children's Hospital of Nanjing Medical University
Ruonan Wang (2020144220@qq.com)
Children's Hospital of Nanjing Medical University
Xuming Mo
Children's Hospital of Nanjing Medical University

Research

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Abstract

Persistent pulmonary arterial hypertension after ventricular septal defect surgery (VSD-PAH) has a severe consequence, and plasma protein profiles in VSD-PAH are unknown.Pulmonary arterial hypertension associated with congenital heart disease (CHD-PAH) has serious consequence and plasma protein profiles in CHD-PAH are unknown. We aimed to reveal the differential plasma proteins in 272 CHD patients with or without PAH. Various types of CHD-PAH were studied. Differential plasma proteins were first detected by iTRAQ proteomic technology and those with significant clinical relevance were selected for further ELISA validation in new cohort of patients.

Among the 190 differential plasma proteins detected by iTRAQ, carbamoyl-phosphate synthetase I (CPSI, related to urea cycle and endogenous nitric oxide production) and complement factor H-related protein 2 (CFHR2, related to complement system and coagulant mechanism) were selected for further ELISA validation in new cohort of 152 patients. Both CPSI and CFHR2 were down-regulated with decreased plasma levels ($p < 0.01$). Thus, we for the first time in CHD-PAH patients identified a large number of differential plasma proteins. The decreased CPSI expression in CHD-PAH patients may reveal a mechanism related to endogenous nitric oxide and the decrease of CFHR2 protein may demonstrate the deficiency of the immune system and coagulation mechanism. The findings may open a new direction for translational medicine in CHD-PAH with regard to the diagnosis and progress of the

disease.

Introduction

Pulmonary arterial hypertension (PAH) is a common complication of congenital heart disease, affecting both disease progression and prognosis(1). It can be developed with the left-to-right shunt and characterized by increased pulmonary vascular resistance and remodeling, leading to severe consequences such as right ventricular failure and death. Unlike preoperative PAH, pulmonary vascular disease may also limit the successful treatment of congenital cardiac defects in pediatric patients. Persistent postoperative pulmonary hypertension is associated with more low survival estimates compared to other etiologies of pulmonary vascular disease in the pediatric population(2). However, the underlying molecular mechanism is unclear.

The proliferation of smooth muscle cells in the small peripheral pulmonary arteries is a common characteristic in all forms of PAH, and the inflammatory mechanism also seems to play an essential role in certain forms of PAH(3). In response to vascular abnormalities, platelets can produce prothrombotic, vasoactive, or mitogenic factors that participate in vasoconstriction and vascular remodeling of idiopathic pulmonary hypertension. In all forms of PAH, the progressive vasculopathy is involved with a significant imbalance of vasodilators, such as nitric oxide (NO) and prostacyclin, and vasoconstrictors, such as endothelin-1 (ET-1) and thromboxane A2(4). This condition likely precedes the development of secondary aberrant cellular proliferation. Classic vasodilator systems are dysregulated with decreases in endothelial NO synthase (eNOS) function caused by enzymatic uncoupling, decreases in the production of prostacyclin (cyclooxygenase-2 dysfunction), and increased abundance and activity of the vasoconstrictor and mitogenic ET-1 signaling system(5). However, there are few reports on proteomics of this clinical syndrome.

Plasma serves as an ideal source of disease biomarkers study because it circulates through, or comes in contact with the majority of organs. During the development of the disease, some proteins are secreted or shed by the organs or tissues, which appear to be the potential biomarker for the disease. We recently used proteomic methods to demonstrate the plasma protein changes in CHD patients that may reveal the possible mechanisms related to the vascular structure are associated with coarctation of the aorta in children(6–7). More recently, we have further reported other protein changes in patent ductus arteriosus patients. We also have, for the first time, identified alterations of 14 differential proteins or polypeptides in the plasma of patients with various valvular heart diseases, which indicate the possible genetic deficiency in these patients.

On the basis of these studies, in the present study, we used DIA proteomic methods to investigate the plasma proteins from a completely new group of patients with CHD-PAH and controls in order to identify the differential proteins related to the pathogenesis of CHD-PAH.

Materials And Methods

Ethical approval

The study and experimental protocols were approved by the Ethics Committee (Institutional Review Board) of the hospital (201705086-1). We confirm that the informed consent was obtained from all subjects-the parents or guardians of the children with congenital heart disease (CHD). In addition to this, we confirm that all methods were performed in accordance with the relevant ethical guidelines and regulations.

Study population

All included patients were diagnosed as non-restrictive ventricular septal defects (> 0.7 cm) associated with PAH with or without atrial septal defects or patent ductus arteriosus. Participants' entry time and the deadline for inclusion were June 2018. and June 2019, respectively. Exclusion criteria: i) patients who had other CHDs, including congenital malformations of cardiac chambers and connections, congenital malformations of aortic and mitral valves, ii) patients with congenital lobar emphysema, bronchogenic cysts, and cystic lung lesions and iii) patients with other major organic diseases, such as severe hepatic diseases, and renal diseases, especially immunological or autoimmune diseases. Of these, a total of 129 patients met the inclusion criteria and were included in the study. After a propensity score-matched (PSM) analysis, two baseline-matched groups (15 pairs, n= 30) were generated (Figure 1). 6 pairs patients randomly selected patient samples were taken for validation. The patients were recruited for the DIA proteomic study were randomized with no bias for selection from two baseline-matched groups. The demographics of the patients in the study of DIA and ELISA are shown in Tables 1 and Tables 2.

Clinical diagnosis

The clinical diagnosis and treatment process of each participant is entirely following the NCCN guideline. The diagnosis was verified by preoperative echocardiography and/or CT angiography, and corrective surgery. The diagnosis of PAH was made on the guidelines by ACCF/AHA11 and European guidelines. According to ACCF/AHA 2009 expert consensus document on pulmonary hypertension11, PAH was defined as mean PAP > 25 mmHg at rest, >30 mmHg during exercise, or systolic PAP > 40 mmHg. In the present study, PAH was defined as either systolic PAP > 40 mmHg or mean PAP ≥ 25 mmHg at rest either by a transthoracic Doppler echocardiography or direct measurement from the main pulmonary artery during surgery and this is in accordance with European guidelines recently published. The PAH was then confirmed during corrective surgery with direct measurement of pulmonary artery pressure. All patients underwent corrective surgery for the heart defects. All surgical procedures were performed by the same surgical team.

Plasma samples

Blood samples were taken from patients before surgery. From each sample, 2 ml blood was harvested 24–48 hours before the day of surgery in collection tubes with EDTA and prepared as described previously13 and then centrifuged at 1500 g for 10 min, the plasma was separated from the blood cells. Plasma was then collected, divided into aliquots, and stored frozen at −80 °C until the analysis was carried out.

Sample Preparation and Fractionation for DDA library Generation

An aliquot from each sample was pooled for DDA library generation. To improve the dynamic range, 100µl of pooled plasma was immunodepleted for removal of the 14 most abundant human plasma proteins using Agilent Multiple Affinity Removal System (MARS) coupled to a High Performance Liquid Chromatography (HPLC, Shimadzu LC-10AT). Protein depletion was undertaken according to the manufacturer's instructions and buffer exchange was performed with 25mM ammonium bicarbonate using spin columns with a 10kDa-molecular weight cut-off (Merck Millipore). Both of high- abundance and low-abundance protein components were collected. The protein concentration was determined using a Bradford Assay according to the manufacturer's (BioRad) instructions.

Protein digestion was performed according to the FASP procedure. Briefly, 200 µg of proteins were incorporated into 30 µl STD buffer (4% SDS, 100 mM DTT, 150 mM Tris[1]HCl pH 8.0). The detergent, DTT and other low-molecular-weight components were removed using UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 30 kD). 100 µl 0.05 M iodoacetamide in UA buffer was added to block reduced cysteine residues. Then, the samples were incubated for 30 min in darkness. After that, the filters were washed with 100 µl UA buffer three times and then 100 µl 25 mM NH4HCO3 twice. Finally, the protein suspensions were digested with 2 µg trypsin (Promega) in 40ul 100mM NH4HCO3 buffer overnight at 37 °C. The resulting peptides were collected as a filtrate. Peptide content was estimated by UV light spectral density at

280 nm.

Digested pool peptides of low-abundance proteins were then fractionated to 10 fraction using Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit. Each fraction was concentrated by vacuum centrifugation and reconstituted in 10 µl of 0.1% (v/v) formic acid. Collected peptides were desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 ml, Sigma) and reconstituted in 40 µl of 0.1% (v/v) formic acid. The iRT-Kits (Biognosys) was added to correct the relative retention time differences between runs with volume proportion 1:3 for iRT standard peptides versus sample peptides.

Data Dependent Acquisition (DDA) Mass Spectrometry Assay

All fractions for DDA library generation, including fractions of low-abundance proteins and high-abundance proteins, DDA runs were injected on a Thermo Scientific Q Exactive HF mass spectrometer connected to an Easy nLC 1200 chromatography system (Thermo Scientific). The peptide (1.5 µg) was first loaded onto an EASY-SprayTM C18 Trap column (Thermo Scientific, P/N 164946, 3 um, 75 um*2 cm), then separated on an EASY-SprayTM C18 LC Analytical Column (Thermo Scientific, ES802, 2 um, 75 um*25 cm) with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of 250 nl/min over 90 min. MS detection method was positive ion, the scan range was 300-1650 m/z, resolution for MS1 scan was 60000 at 200 m/z, target of AGC (Automatic gain control) was 3 e6, maximum IT was 25ms, dynamic exclusion was 30.0s. Each full MS–SIM scan followed 20 ddMS2 scans. Resolution for MS2 scan was 15000, AGC target was 5 e4, maximum IT was 25 ms and normalized collision energy was 30 eV.

Mass Spectrometry Assay for Data Independent Acquisition (DIA)

Each sample peptides were analysed by LC-MS/MS operating in the data-independent acquisition (DIA) mode by Shanghai Applied Protein Technology Co., Ltd. Each DIA cycle contained one full MS–SIM scan, and 30 DIA scans covered a mass range of 350–1650 m/z with the following settings: SIM full scan resolution was 60,000 at 200 m/z; AGC 3e6; maximum IT 50ms; profile mode; DIA scans were set at a resolution of 30,000; AGC target 3e6; Max IT auto; normalized collision energy was 30 eV. Runtime was 90 min with a linear gradient of buffer B (80% acetonitrile and 0.1% Formic acid) at a flow rate of 250 nl/min. QC samples (pooled sample from equal aliquot of each sample in the experiment) were injected with DIA mode at the beginning of the MS study and after every 6 injections throughout the experiment, which was used to monitor the MS performance.

Mass spectrometry data analysis

For DDA library data, the FASTA sequence database was searched with MaxQuant software (version1.5.3.17). The database was downloaded at website: http://www.uniprot.org. iRT peptides sequence was added (>Biognosys|iRT[1]Kit|Sequence_fusion

LGGNEQVTRYILAGVENSKGTFIIDPGGVIRGTFIIDPAAVIRGAGSSEPVTGLDAKTPVISGGPYEYRVEATFGVDESNAKTPVITGAPYEYRDGLDAASYYAPVRADVTPADFS
The parameters were set as follows: enzyme is trypsin, max missed cleavages is 2, fixed modification is carbamidomethyl (C), dynamic modification is oxidation (M) and acetyl (Protein N-term). All reported data were based on 99% confidence for protein identification as determined by false discovery rate (FDR

=N (decoy)*2/(N(decoy)+ N(target))) ≤ 1%. Spectral library was constructed by importing the original raw files and DDA searching results into Spectronaut Pulsar X TM_12.0.20491.4 (Biognosys). DIA data was analyzed with Spectronaut Pulsar X TM searching the above constructed spectral library. Main software parameters were set as follows: retention time prediction type is dynamic iRT, interference on MS2 level correction is enabled, and cross run normalization is enabled. All results were filtered based on Q value cutoff 0.01 (equivalent to FDR<1%).

Bioinformatic analysis

GO and KEGG pathway enrichment analyses.

Proteins fulfilling these criteria were considered to have 'statistical significance'. Blast2GO software was used to get the proteins Gene Ontology (GO) annotation. The expect value <0.001 was used to cut off the Blast result. And the GO term with Blast2GO's score >30 to be consider. The GO annotation of proteins is divided into three major categories: Biological process, cellular component and molecular function. The KEGG database was used for pathway enrichment analysis.Additionally we used KAAS (KEGG Automatic Annotation Server) to acquire KEGG annotations.

The Pathway analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to get protein that identified KO annotations and pathway identify. The Fisher Exact test was used for the Pathway enrichment.Finally, the combined score is a combination of the p-value and z-score calculated by multiplying the two scores as follows:c = log(p) ∗ zwhere c is the combined score, p is the p-value computed using Fisher's exact test, and z is the z-score computed to assess the deviation from the expected rank. PCA (principal analysis component) analysis was performed using Ingenuity Pathway Analysis (IPA) from Qiagen. PCA is a quality controls analysis and it was performed in order to detect outliers and to evaluate the variability within each group.The submitted proteins were first annotated using the KEGG online service tool KAAS (https://www.genome.jp/tools/kaas/)

Cluster analysis based on protein functional enrichment

Cluster analysis based on functional enrichment was used to investigate potential links and differences in specific functions (including GO, KEGG pathways and protein domains). First, the functional classification information and the corresponding P-value was collected and the functional classes that were significantly enriched were then screened out (P<0.05) in at least one protein cluster. The filtered P-value matrix was subjected to a logarithmic transformation of -log10, and the transformed data matrix was then subjected to Z transformation for each functional classification. Finally, the Z-transformed dataset was analyzed by hierarchical clustering (Euclidean distance, average connected clustering). The clustering association was visualized using heatmap.2 in the R package (v3.6.0).

Enzyme-Linked Immunosorbent Assay

In new group of patients, further validation of candidate proteins was performed by using human enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO BIOTECH, Life Sciences Advanced Technologies Inc, USA). By the selection criteria mentioned above, there were two proteins, carbamoyl-phosphate synthetase I and complement factor H-related Protein 2, that were further validated in the plasma. The methods followed the manufacturer's instructions.

Statistical Analysis

Variables following a non-normal distribution were expressed as the median and interquartile range(IQR). Categorical variables were expressed as proportions (percentage). Two-sided t tests and analysis of variance were used to compare means. The chi-square ($\chi 2$) test was used to compare proportions. The Mann–Whitney U test andKruskall–Wallis tests were used to compare medians. For continuous variables, differences between the two groups were evaluated with the unpaired t-test or Mann-Whitney rank test. Data were expressed as mean ± standard deviations. For discrete variables, differences were expressed as counts and percentages, and analyzed with $\chi 2$ or Fisher's exact test between the groups as appropriate. To adjust any baseline potential confounders, propensity score-matched (PSM) analysis was performed using the logistic regression model. The logistic model by which the propensity score (PS) was estimated showed good predictive value (C statistic = 0.75). Patients in the E-ZES group were then matched in a one-to-one manner to those in the I-ZES group according to propensity scores with the nearest available pair matching method.We tested all the available variables that could be of potential relevance; To overcome the limitations of the PSM analysis, we also performed the multivariate analysis. We included only meaningful confounding covariates ($p < 0.05$ or having predictive values) during the multivariable Cox regression analysis, as shown in Table 3. Various clinical outcomes were estimated with the Kaplan-Meier method, and differences between the two groups were compared with the log-rank test. Proportional hazard models were used to compare the hazard ratio of E-ZES with the adjusted PS of I-ZES. For all analyses, a two-sided p value < 0.05 was considered statistically significant.

SPSS 16.0 software (SPSS Inc, Chicago, IL) and GraphPad Prism 5 Demo software (GraphPad Software, San Diego, CA) were used for statistical analysis. When comparison was made among three groups one-way ANOVA and post hoc test were used. Bonferroni test (when equal variances were assumed) or Dunnett's T3 test (when equal variances were not assumed) were used as the post-hoc tests. When appropriate, unpaired t test was used to compare values between two groups. Statistical significance was defined as $P < 0.05$.

Results

Patient characteristics

The demographics of the cohorts are summarized in Tables 1. No significant differences were present among the groups except for the age of atrial septal defect associated with pulmonary arterial hypertension (ASD-PAH) and atrial septal defect (ASD) group compared with controls in iTRAQ proteomic study (Table 1). Similar differences of age between ASD-PAH and Mix group also existed in the ELISA validation study (Table 2).

Proteomic analysis

Mass spectrometry identified 2371 peptides or proteins, of which 1044 were found to be statistically significant, i.e., the proteins fulfilled both the FDR <1% and protein confidence >99% criteria. Of the 1044 proteins, 190 proteins in VSD-PAH group, 185 proteins in ASD-PAH group and 190 proteins in Mix-PAH group were increased or increased (>1.2-fold or <0.833-fold) relative to the control samples. The cut-off value is recommended by literature. Differential proteins expressed in all 6 patient groups with CHD/CHD-PAH compared to the control, identified by iTRAQ were listed in Table 3. In addition, all differentially expressed proteins identified in iTRAQ study are listed in Data file S2. Specifically, eight proteins were significantly lower and two significantly higher in the disease groups. The peptide mass fingerprint spectra are also provided in Table 2. Of these, two proteins (LPA and ANGPTL3) which were associated with PAH were selected for following validation. Compared to CHD, the pathway involved in CHD-PAH with p-value < 0.05 was listed in Table 4. Further, 1009 (i.e., identified in both CHD-PAH and CHD vs. controls) of the identified proteins were used for the ontology analysis (versus 1044). The ontology analysis of the identified proteins indicated the relevance and diversity of molecular functions. With regard to gene ontology (GO) molecular function classification, protein binding (55.5%), catalytic activity (14.6%), and enzyme regulator activity (11.96%) are the categories in which most of the proteins were identified. With regard to GO biological process classification, many of the identified proteins were involved in biological regulation (9.16%), response to stimulus (9.44%), or regulation of biological process (8.56%) (Fig. 1b). Similarly, in GO cellular component classification (Fig. 1c), the identified proteins are related to extracellular region (17.22%), cell (13.35%), or membrane (10.70%).

Validation of the candidate protein by ELISA

To validate the altered plasma proteins LPA and APOC1, ELISA was used to measure plasma levels of these proteins in patients. The demographics of study population in the study of ELISA are shown in Table 2. The plasma LPA level in VSD-PAH patients (109.2 ± 7.718 pg/ml, n = 40; P = 0.000 by ANOVA compared with control group) and VSD patients (196.5 ± 35.68 pg/ml, n = 21; P = 0.000 by ANOVA compared with control group) were significantly lower than that in normal controls (431.8 ± 41.42 pg/ml, n = 37; Fig. 2). Importantly, the LPA protein was also confirmed to be significantly increased in VSD-PAH patients compared with VSD patients (P = 0.012 by ANOVA).

Discussion

The present study for the first time, by using the iTRAQ proteomic technology in CHD-PAH patients, has found that (1) a large number of proteins are altered in the plasma of CHD-PAH patients; (2) increased LPA expression in CHD-PAH patients may reveal a mechanism that is responsible for increased endogenous NO that is a critical pathway in the development of PAH; and (3) the decrease of LPA protein, that may possibly activate the complement cascade, in the plasma of CHD-PAH patients may demonstrate the deficiency of the immune system and coagulation mechanism in these patients.

Recently, proteomic methods have been used to investigate the protein changes in hereditary PAH that used 2-dimensional PAGE in combination with liquid chromatography/tandem mass spectrometry analysis17. To our knowledge, the present study is the first proteomic study on the plasma of CHD-PAH patients by using iTRAQ technology.The present study provides potentially important insights into plasma proteome changes in CHD-PAH. We found abundant differentially expressed plasma proteins associated with molecular function, biological processes, and cellular components. The involved various biological processes include extracellular matrix, binding, catalytic activity, and biological regulation (Fig. 1). According to gene ontology (GO) analysis between CHD-PAH and CHD, several genes are involved in blood coagulation, hemostasis, wound healing, cytoskeletal protein binding, and platelet activation (Table S2.). The analysis suggests that pulmonary arterial remodeling in PAH are likely caused by different molecular mechanisms and may require specific therapeutic options.On the basis of our iTRAQ results, we compared the plasma proteins among CHD patients with or without PAH and the normal controls. In the present study, we have detected ~190 differential proteins in different types of CHD-PAH patients in comparison to the control. Most of them referred to innate immunity, inflammation and tissue injury, and platelet or coagulation proteins.

Among the 10 differentially expressed proteins in the plasma of CHD-PAH and CHD patients in comparison to the control, we chose two of them (LPA and APOC1) for further validation that were also expressed differently between the specific CHD with/without PAH. In fact, these two proteins also have clear implications in the pathology of PAH. LPA is related to the urea cycle that regulates NO production (see Fig. 4 for the mechanism). In contrast, APOC1 is related to the complement system and therefore is related to immune mechanism. The down-regulated expression of LPA was validated in new cohort of patients with various types of CHD-PAH and the results clearly showed that LPA was altered in all CHD-PAH patients. In particular, in VSD-PAH and VSD patients compared to the control, LPA was increased in VSD and further increased in VSD-PAH patients, suggesting that LPA may serve as an important biomarker for CHD-PAH.

LPA is a mitochondrial enzyme that is related to the urea cycle as mentioned above. Endogenous NO is critical for the maintenance of normal pulmonary arterial pressure and is derived from arginine supplied by the urea cycle(8). The rate-limiting step in the urea cycle is catalyzed by the mitochondrial enzyme LPA. Vascular endothelial cells synthesize endogenous L-arginine by recycling L-citrulline, using argininosuccinic acid synthase and lyase and convert L-arginine via nitric oxide synthase (NOS) to L-citrulline and NO. As well known, NO induces vasodilation, inhibits platelet aggregation and leukocyte adhesion, inhibits vascular smooth muscle cell proliferation, and modulates oxidative stress(9). Therefore, the altered LPA is possibly related to the alteration of the above biological processes through reduction of the NO production. In fact, polymorphism in the gene encoding LPA has been suggested to influence NO production as well as vascular smooth muscle reactivity and the LPA T1405N genotype appears to be an important new factor in predicting susceptibility to increased pulmonary artery pressure following surgical repair of congenital cardiac defects in children27. Our study is the first that reveals the LPA protein alteration in CHD-PAH patients. Our study also has a particularly important clinical implication because the LPA protein alteration in CHD-PAH patients was found in the plasma of the patients. The lower plasma concentration of LPA in CHD-PAH patients seems to reflect the impaired NO production which is important in maintaining PAH, because more subtle changes in carbamoyl-phosphate synthetase affect the availability of arginine and citrulline22 that are involved in the production of NO. Indeed, the present study suggests that the plasma LPA level may reflect the pathology of PAH and the significant decrease of plasma LPA level may indicate the presence of PAH. Further, the plasma LPA level may be developed as a biomarker of PAH.

Interestingly, compared with CHD, the pathways involved in CHD-PAH also shed light on the understanding of the factors contributing to the progress of PAH, such as extracellular matrix (ECM)-receptor interaction, focal adhesion, chemokine signaling pathway, and complement and coagulation cascades(10). Some of the pathways have been previously identified in PAH. For example, chemokines produced from small pulmonary artery of PAH patients appear to contribute to inflammatory cell recruitment and smooth muscle cell proliferation33. In addition, changes in ECM protein abundance in PAH have been reported. For example, It has been shown that human plasma levels of TIMP-4, tenascin-C, MMP-2, and NT-proBNP, all ECM interacting proteins(11), are elevated in PAH patients and are correlated with the severity of the disease34. In this study, several altered proteins are involved in the ECM-receptor interaction, such as collagen, thrombospondin (THBS), fibronectin, vitronectin, and von Willebrand Factor (vWF), all of which were increased in the plasma of CHD-PAH patients (Fig. S11). Tissue remodeling is generally characterized by extensive fibrosis and additional changes in the expression of cardiac ECM-associated proteins35,36,37 and the cardiac ECM provides structural support and facilitates mechanical, electrical, and chemical signals during homeostasis and in response to stress or injury38,39,40(12-14). Together with the findings previously reported, ECM factors play an important role in the remodeling referred to the process of PAH. This, however, need to be further studied in the future.

Limitations

Our study has some limitations. Due to the fact that patients with ASDs develop PAH at later stages of the disease, in the present study, patients with ASD and PAH inevitably had older age compared to the patients with ASD without PAH and to the control subjects, although the age of other groups was matched with the control. This could be the reason for no significant differences of the level of candidate proteins between ASD or Mix patients with or without PAH in the validation. In addition, proteomic studies reveal abundant unnamed protein products as appeared in the differential protein list of the present study. Most of them are characterized by highly similar to some particular proteins. For example, a down-regulated protein with accession is described as highly similar to thrombospondin. In fact, thrombospondin-1 is hypoxia-responsive mitogens that promote vascular smooth muscle cell proliferation as the critical process in the pathogenesis of pulmonary hypertension41. These candidate proteins remain to be further studied. In addition, FVIII and vWF are well known to be involved in endothelial dysfunction that is a key process in the pathogenesis of PAH. Due to our selection criteria mentioned before, these proteins were not of priority for validation in the present study.

In conclusion, the present study for the first time, by using the iTRAQ proteomic technology in CHD-PAH patients, identified a large number of differential plasma proteins. The increased LPA expression in CHD-PAH patients may reveal a mechanism that is responsible for increased endogenous NO in the development of PAH and the decrease of APOC1 protein may demonstrate the deficiency of the immune system and coagulation mechanism in these patients.

Declarations

Author Contributions

Di Yu performed the experiments, analyze data, write up the manuscript; Xiaodong Zang collected samples and help experiments; Ruonan Wang collected samples and prepare the manuscript; Xuming Mo designed the study and protocol, collect samples, analyze data, discussion on the results, and write up the manuscript.

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Availability of data and material

Please contact the author for data requests.

Declarations

Ethics approval and consent to participate

Study approval and ethical clearance were obtained from the Institutional Ethical Committees of Children’s Hospital of Nanjing Medical University (201705086-1).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Tables

Clinical characteristics	Total study population			Propensity-matched patients		
	VSD-non-PH	VSD-PH	P	VSD-non-PH	VSD-PH	P
N	129	20		15	15	
Male, n(%)	67 (51.94)	6 (30.00)	0.09	7 (50)	5 (35.7)	0.70
Age (month)	10.43 ± 21.79	14.79 ± 19.67	0.12	8.23 ± 12.30	9.59 ± 7.13	0.72
Weight (kg)	7.05 ± 3.48	7.54 ± 3.61	0.28	6.23 ± 1.88	6.96 ± 2.15	0.34
Defect diameter (mm)	11.18 ± 2.71	12.84 ± 3.94	0.09	9.61 ± 1.83	12.05 ± 3.41	0.02
Preoperative PAP (mmHg)	56.73 ± 12.57	65.75 ± 18.63	0.05	56.93 ± 12.93	58.29 ± 14.13	0.79
Oxygen saturation (%)	97.57 ± 2.04	97.30 ± 1.66	0.14	97.65± 1.96	97.36 ± 1.64	0.25
Heart rate (beats/min)	131.60 ± 16.23	131.80 ± 15.58	0.91	133.21 ± 17.09	133.29 ± 16.87	0.99
Systolic pressure (mmHg)	95.32 ± 15.33	96.62 ± 10.97	0.81	104.48 ± 17.26	97.82 ± 12.69	0.25
Aalbumin (g/l)	45.00 ± 5.62	46.70 ± 5.44	0.19	44.29 ± 6.34	46.36 ± 5.31	0.35
Hemoglobin (g/l)	114.74 ± 23.01	119.50 ± 12.01	0.01	113.14 ± 10.90	115.57 ± 11.86	0.57
Red blood cell count ($x10^{12}$/L)	4.22 ± 0.58	4.38 ± 0.48	0.12	4.18 ± 0.57	4.29 ± 0.52	0.61
Platelet count ($x10^{9}$/L)	338.37 ± 102.92	316.40 ± 109.77	0.21	347.36 ± 94.20	328.50 ± 122.02	0.65
CPB time (min)	51.83 ± 13.64	61.85 ± 29.59	0.20	47.71 ± 11.58	53.50 ± 15.01	0.26
ACC time (min)	31.92 ± 10.93	34.96 ± 12.28	0.40	28.96 ± 6.95	32.06 ± 11.03	0.38
ASD, n(%)	91 (70.54%)	13 (65.00%)	0.60	10 (71.4)	8 (57.1)	0.69
Tricuspid regurgitation, n(%)			0.51			1
No regurgitation	2 (1.55%)	0 (0.00%)		0 (0)	0 (0)	
Mild regurgitation	125 (96.90%)	19 (95.00%)		13 (92.9)	14 (100)	
Moderate regurgitation	2 (1.55%)	1 (5.00%)		1 (7.1)	0 (0)	
Mitral regurgitation, n(%)			0.35			1
No regurgitation	10 (7.75%)	0 (0.00%)		1 (7.1)	0 (0)	
Mild regurgitation	119 (92.25%)	20 (100.00%)		13 (92.9)	14 (100)	
Phosphodiesterase-5 inhibitor, n(%)	110 (85.27%)	19 (95.00%)	0.31	12 (85.7)	13 (92.9)	1
Endothelin antagonist, n(%)	3 (2.33%)	4 (20.00%)	0.01	1 (7.1)	0 (0)	1

All patients (n =149)	Statistics	Univariate analysis		Multivariate analysis	
		Odds ratio	P value	Odds ratio	P value
Sex		1			
Male	73 (48.99%)	2.52 (0.91, 6.97)	0.0746		
Female	76 (51.01%)	1.01 (0.99, 1.02)	0.4155		
Age (month)	11.02 ± 21.50	1.04 (0.92, 1.16)	0.5591		
Weight (kg)	7.11 ± 3.49	1.19 (1.02, 1.38)	0.0227	1.05 (0.91, 1.20)	0.5166
Defect diameter (mm)	11.40 ± 2.94	1.05 (1.01, 1.08)	0.0088	1.05 (0.96, 1.14)	0.2868
Preoperative PAP (mmHg)	57.94 ± 13.81	1.00 (0.97, 1.03)	0.9596		
Heart rate (beats/min)	131.63 ± 16.09	1.01 (0.97, 1.04)	0.7124		
Systolic pressure (mmHg)	95.49 ± 14.79	0.94 (0.75, 1.17)	0.5673		
Oxygen saturation (%)	97.54 ± 1.99	1.06 (0.97, 1.15)	0.2093		
Aalbumin (g/l)	45.23 ± 5.61	1.01 (0.99, 1.02)	0.3869		
Hemoglobin (g/l)	115.38 ± 21.89	1.67 (0.68, 4.14)	0.2639		
Red blood cell count (x1012/L)	4.24 ± 0.56	1.00 (0.99, 1.00)	0.3772		
Platelet count (x109/L)	335.42 ± 103.75	1.03 (1.00, 1.05)	0.0326	1.00 (1.00, 1.00)	0.1685
CPB time (min)	53.17 ± 16.89	1.02 (0.98, 1.06)	0.2581		
ACC time (min)	32.33 ± 11.12	0.68 (0.26, 1.77)	0.4254		
Arrhythmia	72 (48.32%)	0.78 (0.29, 2.10)	0.6161		
ASD	104 (69.80%)	1.01 (0.96, 1.05)	0.7531		
ANGPTL3 (mg/dl)	29.74 ± 10.05	10.43 (3.78, 28.79)	<0.0001	1.01 (0.99, 1.03)	0.2246
APOC1 (mg/dl)	29.60 ± 9.19	1.24 (1.14, 1.36)	<0.0001	1.00 (0.98, 1.02)	0.9353
LPA (mg/dl)	3.55 ± 1.01	**1.81 (1.15, 2.85)**	**0.0099**	**1.61 (1.28, 2.04)**	**<0.0001**
Phosphodiesterase-5 inhibitor	129 (86.58%)	10.50 (2.15, 51.22)	0.0036	**5.63 (1.90, 16.75)**	**0.0019**
Endothelin antagonist	**7 (4.70%)**	**0.94 (0.75, 1.17)**	**0.5673**		

Figures

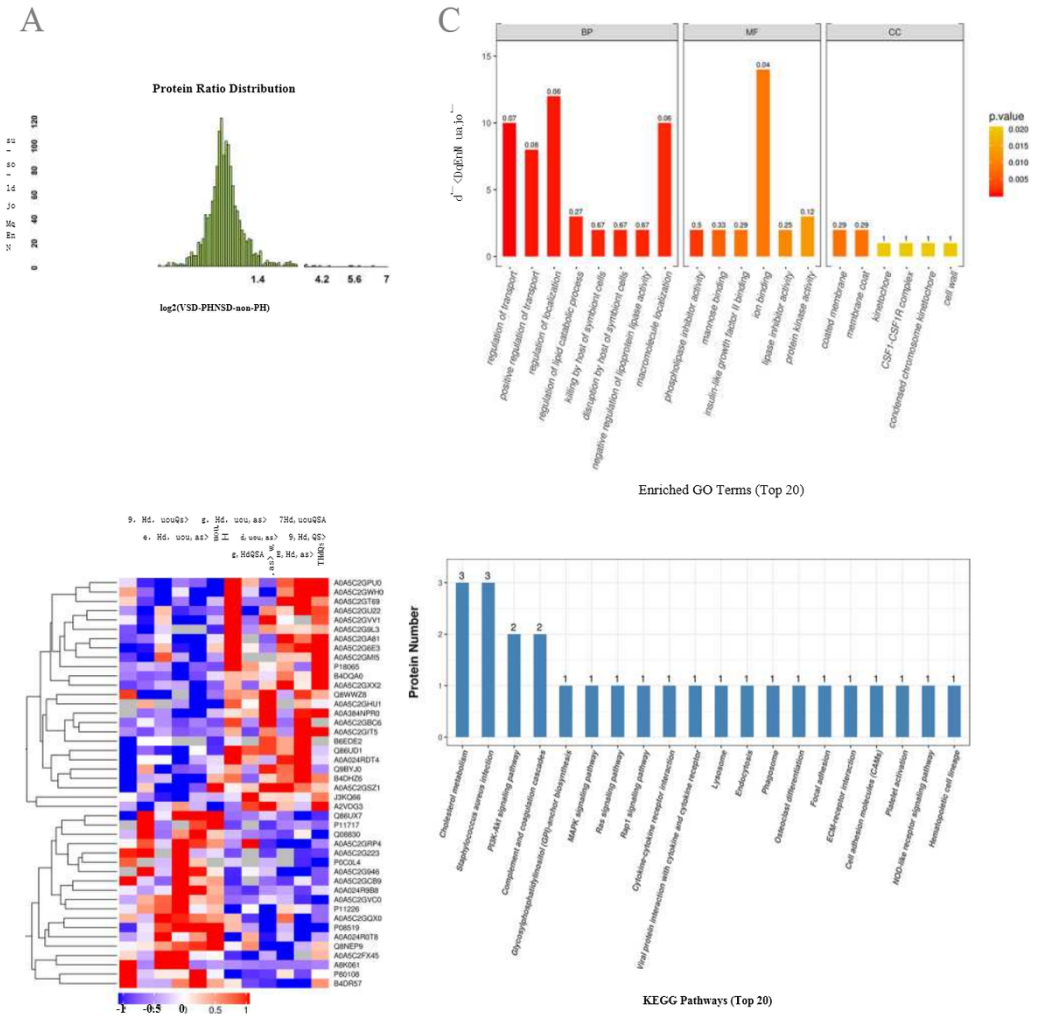

Figure 1

propensity score-matched (PSM) analysis, two baseline-matched groups (15 pairs, n= 30) were generated (Figure 1).