

iTRAQ-based Quantitative Proteomic Analysis of Thoracic Aortic from Adult Rats Born to Pre-eclamptic Dams

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Abstract

Background: Preeclampsia and gestational hypertension can cause impaired vascular function in offspring. In our previous work, we described the profiles of protein expression in umbilical artery tissues of patients with preeclampsia.

Methods: In order to gain insights into the mechanisms of vascular dysfunction in adult rats born by preeclampsia dams, we present analysis of thoracic aortic tissues by using iTRAQ isobaric tags and 2D nano LC-MS/MS.

Results: By using iTRAQ method, we analyzed 1825 proteins in which 106 proteins have shown significantly differential expression in thoracic aortic. Results from ingenuity pathway analysis (IPA) showed majority of proteins with differential expression (DEP) were associated with cardiovascular function. Further analysis indicated that glucose-6-phosphate dehydrogenase (G6PD), which is inhibited by miR-423-5p and activated by TP53, was most affect the cardiovascular function. The expression level of G6PD was up-regulated in thoracic aortic tissues as confirmed by western blot results. Two other vascular function proteins Cysteine and glycine-rich protein 2 (CSRP2) and Tubulin alpha-4A (TUBA4A) were upregulated as demonstrated by mass spectrometry (MS).

Conclusions: Although the results need further functional validation, these data provide novel findings relating to impaired vascular function in adult offsprings of preeclamptic mothers.

1. Introduction

Preeclampsia, a major medical pregnancy complication, characterized by high incidence of fetal and maternal mortality [1]. Previous studies defined the condition as *de novo* elevation of blood pressure (BP) to > 140/90 mmHg past the 20th week of gestation, and this is followed by proteinuria (> 0.3 g/24h or \geq + 1 on dipstick) [2, 3]. Earlier studies had been shown that women who develop preeclampsia are predisposed to cardiovascular diseases [4, 5]. Interestingly, recent epidemiological studies have suggested preeclampsia not only affects the mother, but also the children [6–8].

In fact, a number of reports indicate that preeclampsia would lead to an increase risk of cardiovascular disease in the offspring. In 9-year-old offspring, Geelhoed *et al.* [9] demonstrated a correlation between gestational hypertension and preeclampsia with elevated diastolic and systolic blood pressures. On the other hand, Tenhola *et al.* [10] found that 12-year-old offsprings of preeclamptic mothers exhibited significantly elevated casual diastolic and systolic BP relative to corresponding controls from normal mothers. Moreover, Seidman *et al.* [11] found no evidence linking maternal pre-eclampsia with abnormal cognitive performance and growth in late adolescent children, with the condition linked to hypertension. Kajantie *et al.* [12] reported a significant association between preeclampsia and increased risk of stroke, with severe preeclampsia also linked to incidence of hypertension in adult offspring aged between 60–70 years. To date, however, the molecular mechanisms underlying this phenomenon remains elusive.

In our previous work, we revealed the profiles of proteins derived from the umbilical artery tissue of preeclamptic patients [18]. Herein, we sought to elucidate the drivers of vascular dysfunction in adult rats born by preeclampsia dams. Specifically, we performed analysis of thoracic aortic tissues utilizing 2D nano LC-MS/MS and iTRAQ isobaric tags, followed by quantitative proteomics analysis of thoracic aortic tissues from adult rats (1-year-old) born to pre-eclamptic dams. Our findings provide new knowledge on vascular impairment in adult rats of preeclamptic dams.

2. Materials And Methods

2.1 Animal Model

Animal handling protocols were sanctioned by the Zhejiang University Committee on Animal Care and Use, while experiments adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats (6 to 8 wk old) were procured from Experimental Animals Center of Zhejiang University (Hangzhou, China) and housed in our laboratory under standard conditions and freely provided with food and water. A preeclampsia model was developed as before [13–16]. Briefly, 10 pregnant rats were divided into two groups as follows: one group were treated with L-NAME (50 mg/kg) while the controls received saline via osmotic minipumps starting at day 17 of gestation until day 7postpartum. A total of 22 and 24 controls and L-NAME-exposed pups were born, respectively, and weaned at 21 days. Thoracic aortic of rats from two different groups were dissected, and blood was removed by rinsing. Thoracic aortic specimen were frozen at -80°C .

Determination of blood pressure in 1-year-old rats was done with the tail-cuff method. Approximately 2-mm segments of mesenteric arteries (in the second-order branch of the superior mesenteric artery) were cut to remove fats and connective tissues, followed by mounting on a wire myograph chamber (DMT 620M; Aarhus N, Denmark), according to the manufacturer's recommendations. Briefly, arterial vessel segments were soaked in physiological Krebs' buffer that had been passed through 95% oxygen/ 5% carbon dioxide (pH 7.4), and incubated at 37°C . The contents were maintained in an equilibration period for 30 min, vessel tension elevated to 1 mN, followed by establishment of resting tension for 30 min. The KPSS was used to cause maximum contraction of the mesenteric arteries, which were then subjected to varying concentrations of vasoconstrictor phenylephrine (Phe) (10^{-9} to 10^{-5} mol/L). The resulting contraction was presented as % of contraction by 120 mM. Thereafter, a stable contraction plateau was established by Phe ($1\text{ }\mu\text{M}$)-treatment of mesenteric arterial rings to determine acetylcholine-induced vasodilatation. Subsequently, they were treated with varying concentrations of an actylcholine (10^{-8} to 10^{-5} mol/L). The readings are displayed as a % of precontraction relative to Phe.

2.2 Sample Preparation

Approximately 100 mg of thoracic aortic specimen were first crushed to form a fine powder, under liquid nitrogen, then mixed with a buffer comprising 4% SDS, 1 mM DTT, and 150 mM Tris-HCl (pH 8.0), to extract total proteins. The BCA protein assay (Pierce, Rockford, IL, USA) was carried out to quantify the amount of proteins extracted.

2.3 Protein Digestion and iTRAQ Labeling

Proteins were digested using a previously described protocol [17, 18]. About 200 μg of total protein samples were suspended into 30 μL of the aforementioned extraction buffer, supplemented with 100 mM dithiotreitol solution, and subjected 95°C heating for 5 minutes. Sample were cooled subjected to ultrafiltration process (cutoff 10 kDa, Sartorius, Goettingen, Germany) with 200 μL UT buffer (150 mM Tris-HCl, and 8 M Urea, pH 8.0). They were then subjected to centrifugation for 30 min at 14,000 g at 20°C. Next, reduced cysteines were blocked with 100 μL of 50 mM iodoacetamide dissolved in UT buffer), and then subjected to a 20-min incubation in darkness. The resultant filtrate was centrifuged at 14,000 g for 20 min at 20°C, and the washed two times with 100 μL UT buffer at 14,000 g for 20 min. Subsequently, the filtrate was mixed with 100 μL dissolution buffer (AB Sciex, Framingham, MA, USA), centrifuged at the aforementioned speed and temperature for 30 min. This procedure was carried out twice. The obtained filtrate was incubated overnight with 2 μg trypsin in 40 μL of trypsin at 37°C. The filtered units were separately put into fresh tubes and spun at 14,000 g at 20°C for 30 min, then peptide concentrations determined using UV light spectral density at OD280 [19].

We labeled the resultant peptide mixture with 8-plex iTRAQ chemical (AB Sciex, Framingham, MA, USA). Four thoracic aortic specimens belonging to control group (C) were labeled with a mass of 113, 114, 115 and 116 isobaric iTRAQ tags, while four corresponding preeclamptic tissues were labeled with mass 117, 118, 119 and 121 isobaric iTRAQ tags. The specimens, together with labeling solution, were for 2 h at room temperature prior to subsequent characterization.

2.4 Strong Cationic-exchange Chromatographic Separation

This procedure was carried out according to a previous protocol [18]. Briefly, we acidified the combined sample using 1% trifluoroacetic acid, then using a PolySULFOETHYL column (4.6 \times 100 mm, 5 μm , 200 Å, Poly LC Inc., Columbia, MD, USA), it was exposed to strong cationic-exchange chromatography (SCX) fractionation. Solvent A contained 10 mM KH_2PO_4 in 25% (v/v) ACN, whereas solvent B contained solvent A enriched with 500 mM KCl. Application of solvent A and B was done at the following gradients: 0–10% solvent B for 2 min, 10–20% solvent B for 25 min, 20–45% solvent B for 5 min, and 50–100% solvent B for 5 min. The collected fractions (at each minute) were analyzed by measuring the absorbance at 214 nm. The final step involved combining all samples to form 10 fractions, according to the quantity of peptides, followed by desalting using C18 cartridges (Sigma). Each SCX salt step fraction was dried under vacuum centrifugation, after which it was suspended in 40 μL 0.1% (v/v) trifluoroacetic acid.

2.5 LC - ESI- MS/MS analysis

LC-ESI- MS/MS Analysis, described previously [18], was carried out with 5 μg of peptide mixtures from each fraction analyzed using nano LC-MS/MS. Summarily, peptide mixtures were loaded into a Thermo EASY-nLC column, measuring 100 mm \times 75 μm , 3 μm , (Thermo Finnigan, San Jose, CA, USA) in solvent C (0.1% Formic acid), and separated using a linear gradient comprising solvent D (80% acetonitrile with

0.1% (v/v) formic acid) at a flow rate of 300 nL/min over 120 min: 0–100 min with 0–45% solvent D; 100–108 min with 45–100% solvent D; 108–120 min with 100% solvent D.

Data in the positive ion mode was acquired via the Q-Exactive (Thermo Finnigan, San Jose, CA, USA) mass spectrometer, at a selected mass range of 300–800 mass/charge (m/z). Moreover, dynamic exclusion was used with 40.0 s duration, while Q-Exactive survey scans were set at resolutions of 70,000 and 17,500 at m/z 200 for HCD spectra. MS/MS data was obtained using the data-dependent acquisition method, targeting the top 10 most abundant precursor ions. Normalized collision energy was set at 30 eV whereas the underfill ratio on the Q-Exactive was defined as 0.1%.

2.6 Protein identification and quantification

Proteins were identified and quantified using a previously reported protocol [18]. Briefly, proteins were first detected using the MASCOT search engine (version 2.2.1; Matrix Science, London, UK), embedded in the Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA, USA). This was achieved by searching the Uniprot database of rat protein sequences (08-2013, downloaded from: <http://www.uniprot.org/>) as well as a decoy database, using the following search parameters: monoisotopic mass, peptide mass tolerance of ± 20 ppm; fragment mass tolerance of 0.1 Da; trypsin as the target enzyme; and allowing up to two missed cleavages. iTRAQ 8-plex labeled tyrosine and methionine oxidation were considered as variable modifications, whereas fixed modifications were considered as carbamidomethylation on cysteine, N-term of peptides labeled by iTRAQ 8-plex, and lysine. We set the False discovery rate (FDR) for both protein and peptide identification at less than 0.01, and this detection was supported by at least one unique peptide identification.

2.7 Bioinformatics analyses

We carefully analyzed significant differentially expressed proteins ($p < 0.05$), then selected and retained those with differential expression ratios above ± 1.2 . We then used hierarchical cluster analysis, based on Cluster 3.0 and Java Treeview softwares, to determine the value of the resulting DEPs in differentiating our two experimental.

Thereafter, we performed disease and pathway analyses, as well as network generation, using the Ingenuity Pathway Analysis (IPA) software (QIAGEN, Redwood 185 City, CA), a Generally, IPA database that relies on available publications describing biological mechanisms, interaction and functions of protein. Here, calculating z-scores can be used to infer activation states (“inhibited” or “activated”) of related biological processes. The Fisher’s exact test was employed to determine p -values, hence predict the likelihood of association among proteins in some datasets. The resulting biological process was only attributed to chance.

2.8 Western blot assay

Determination of protein expression was performed using Western blot assay as previously described [18]. Briefly, proteins were extracted by homogenizing thoracic aortic tissues in 500 μ L 1 \times RIPA buffer enriched with protease inhibitors (1 μ g/mL phenylmethylsulfonyl fluoride and 1 μ g/mL leupeptin).

Proteins were resolved and transferred onto a nitrocellulose membrane using standard procedures. The membranes were incubated for 1 h with blocking buffer, then overnight with primary antibodies against G6PD (Cell Signaling Technology 12263, Danvers, MA, USA, 1:1000), CSRP2 (Abcam ab178695, Cambridge, UK; 1:1000), TUBA4A (Sangon D110022, Shanghai, China, 1:1000) and β -Actin (Santa Cruz Biotechnology sc1616, Santa Cruz, CA, 1:1000) at 4°C overnight. Finally, the membranes were probed with secondary antibody (1: 5000) at room temperature for 1 h. The membranes were washed thrice, then protein intensities determined using the Odyssey® Imager system (LI-COR, Lincoln, NE, USA).

2.9 Statistical analysis

The GraphPad Prism 6 software (San Diego, CA) was utilized for data analysis. The Student's *t*-test was applied in group comparisons. Data are shown as means \pm standard deviations (SD). Data followed by *p* < 0.05 were considered statistically significant.

3. Results

3.1 Rat model of preeclampsia

The blood pressure was comparable between the two groups after 1 year (Fig. 1A). No significant changes were observed in half year, although the opposite was observed in one-year olds. We found no significant differences in contractile response of mesenteric arteries to phenylephrine between adult rats born to pre-eclamptic mothers relative to controls (Fig. 1B). However, this group exhibited significantly lower relaxation responses to acetylcholine (an endothelium-dependent vasodilator). logEC₅₀ are enumerated in the table.

We evaluated whether proteomic changes in rat thoracic aortic tissues were related to preeclampsia. Untargeted proteomic analysis detected 1825 non-redundant proteins in rat thoracic aortic tissues (Supplemental Table 1). Overall, 106 proteins showed differential expression between preeclamptic group and controls, of which 75 and 31 proteins were significantly upregulated and downregulated, respectively (Supplemental Table 2). Hierarchical clustering of DEPs is illustrated using a heat map (Fig. 2A).

3.2 Validation of protein expression

We validated the profile of protein expression in iTRAQ analysis above using western blot assay, with special focus on the correlation between the differentially expressed proteins with cardiovascular functions. Our blots showed that expression of G6PD, CSRP2, and TUBA4A, which affect cardiovascular function, was significantly (Fig. 2B), which corroborated the findings from iTRAQ analysis.

3.3 Disease and Functional analysis

To reveal the function of the identified DEP, we subjected the expression data to Ingenuity Pathway Analysis, to obtain a scale of significance of association. The DEP were linked to “Disease and Disorder”, as well as “Physiological System Development and Functions”. Moreover, overlapping *p*-values indicated

that these proteins were markedly associated with 24 subcategories of “Disease and Disorder” (Supplemental Fig. 1), and 19 subcategories of “Physiological System Development and Functions” (Supplemental Fig. 2).

Results from “Disease and Disorder” analysis further revealed that 22 proteins were associated with cardiovascular disease (Fig. 3), especially in occlusion of artery, atherosclerosis, coronary disease, coronary artery disease and myocardial infarction. Further, 20 proteins were described to be linked to cardiovascular functions (Fig. 4), especially in angiogenesis, vasculogenesis, abnormal morphology of heart, abnormal morphology of cardiovascular system, and morphology of cardiovascular system.

3.4 Upstream analysis

According to IPA, the term “upstream regulator” is used to refer biomolecules with the potential to influence expression of another biomolecule. Previous studies have shown that upstream regulators can be drugs, chemicals, kinases, receptors, microRNAs, cytokines, and transcription factors. Results of the present study revealed that of the 20 proteins associated with cardiovascular functions, miR-423-5p and TP53 were upstream regulators, miR-423-5p inhibited expression of ANGPTL2, G6PD, LASP1, PXN and SERPIND1, while TP53 mediated activation of CD47, DLG1, G6PD, LASP1, NDRG1, POSTN and S100A4 expression (Fig. 5).

4. Discussion

Proteomics, which refers to functional analysis of all expressed proteins in an organism, has been widely used to complement other functional genomics approaches. Bioinformatics analysis of these datasets can lead to development of a comprehensive gene function database thereby providing useful tools for building and testing of research hypotheses. Previous studies have shown that mass spectrometry-based proteomics have high medical value [20]. Of note, iTRAQ is used to characterize proteins. In our previous studies, we generated profiles of proteins in human umbilical arteries derived from preeclamptic, *in vitro* fertilization (IVF) and ovarian hyperstimulation syndrome (OHSS) patients [18, 21, 22]. Interestingly, our results indicated that these proteins are differentially expressed relative to controls, and are highly correlated with cardiovascular physiology. Previous studies aim to gain reveal the events leading to infant cardiovascular diseases. In this study, we analyzed thoracic aortic from adult rats born by pre-eclamptic dams and identified the proteins related to cardiovascular function.

Herein, the association of protein expression with disease and functions was examined, We found that 22 DEPs were significantly associated with cardiovascular diseases. Additionally, we found that occlusion of artery and atherosclerosis are the most significant disorders, as evidenced by their significant positive correlation with 16 differentially expressed proteins. Among them, transgenic APOA2 protein affects development of atherosclerotic lesion in mouse model [23, 24]; SGCG protein decreases coronary artery spasm in mouse[25]. Knockout SERPIND1 gene in mouse increases formation to atherosclerotic plaque in mouse aortic root [26]. Knockout Pon1 gene increases atherosclerosis in mouse [27]. Mutant human

EIF4H gene is associated with coronary artery disease in human [28]. In human peripheral blood monocytes, the expression of CD47 protein in detergent-resistant membranes was linked to myocardial infarction and coronary artery disease in human [29]. In mouse, knockout ITGB3 gene increases atherosclerosis of aorta [30].

In the analysis of cardiovascular function, we found that 20 proteins are related with cardiovascular function, and angiogenesis was the most affected one. In these proteins, Mouse Hrg decreases angiogenesis of blood vessels[31]. A dominant negative protein fragment (306–423) containing a DNA binding domain from mouse AP1S2 protein decreases angiogenesis in mouse ear [32]. NDRG1 protein increases angiogenesis in tumors derived from NUGC-3 cells from mouse subcutaneous fascia[33]. G6PD (Glucose-6-phosphate dehydrogenase) modulates VEGF-mediated angiogenesis[34]. Interference of human SARS mRNA by siRNA increases sprouting of cultured HUVECs in cell culture[35]. Human DLG1 is involved in the proliferation of endothelial cells [36]. Rat ITGB3 increases angiogenesis of blood vessels[37]. In mouse, knockout ITGA1 gene () decreases angiogenesis of blood vessels in tumor [38]. ANGPTL2 protein increases angiogenesis in mouse [39]. S100A4 protein could act as an angiogenic factor [40]. KLKB1 protein is involved in angiogenesis in mammal [41]. Based on these findings, it is evident that ectopic expression of these proteins can exacerbate susceptibility to cardiovascular disease. However, the precise relationship between these proteins with fetal origins of adult cardiovascular disease remains unclear, necessitating further investigation.

Upstream Regulator Analysis (UPA) has been extensively used to detect upstream regulators that control gene expression changes across experimental datasets. Functionally, IPA allows prediction of activated or inhibited upstream regulators, thereby revealing upregulated and downregulated genes in a dataset. Knowledge of this regulatory cascade allows accurate elucidation of biological activities that may occur in tissues or cells. During upstream analysis of cardiovascular function, miR-423-5p has been implicated in inhibition of G6PD and LASP1 activity, while TP53 was shown to activate G6PD and LASP1 expression. On the other hand, miR-423-5p is a potential biomarker for diagnosis and prognosis of heart failure [42], whereas TP53 has been associated with changes in diastolic blood pressure [43]. Therefore, upstream analysis represents a feasible approach for future studies.

Previous proteomics studies, targeting the human umbilical artery, have reported DEPs identified in the present study play key roles in the cardiovascular system. Both results indicate that preeclampsia could lead to offspring with vascular dysfunction, especially in angiogenesis. And some DEPs were related with cardiovascular disease. Therefore, we can draw conclusion that preeclampsia could affect cardiovascular system in offspring.

5. Conclusion

In conclusion, 24 DEPs were related to cardiovascular disease and 22 DEPs were described to be associated with cardiovascular functions, especially in atherosclerosis and angiogenesis. It is crucial to determine whether the DEPs observed herein are the cause or the effect of cardiovascular functions of

offspring from preeclamptic mothers. Particularly, unraveling the relationship between proteins participating in cardiovascular processes could provide useful revelations into the pathological events underlying vascular diseases.

6. Declarations

- **Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Shaoxing Maternity and Child Health Care Hospital (Ethical Application Ref: 2018022).

- **Consent for publication**

Not applicable.

- **Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the iProX partner repository [44] with the dataset identifier PXD024811.

- **Competing interests**

The authors declare there are no competing interests.

- **Funding**

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- **Authors' contributions**

Bin Yu: Writing - original draft, Hong-Dan Zhu: Methodology, Xiao-Liang Shi: Formal analysis, Pan-Pan Chen: Visualization, Xiang-Mei Sun: Validation, Gui-Yu Xia, Yong-Xing Zhong: Methodology, Min Fang, Xiao-Li Tang: Data curation, Hai-Tao Pan, Tao Zhang: Project administration

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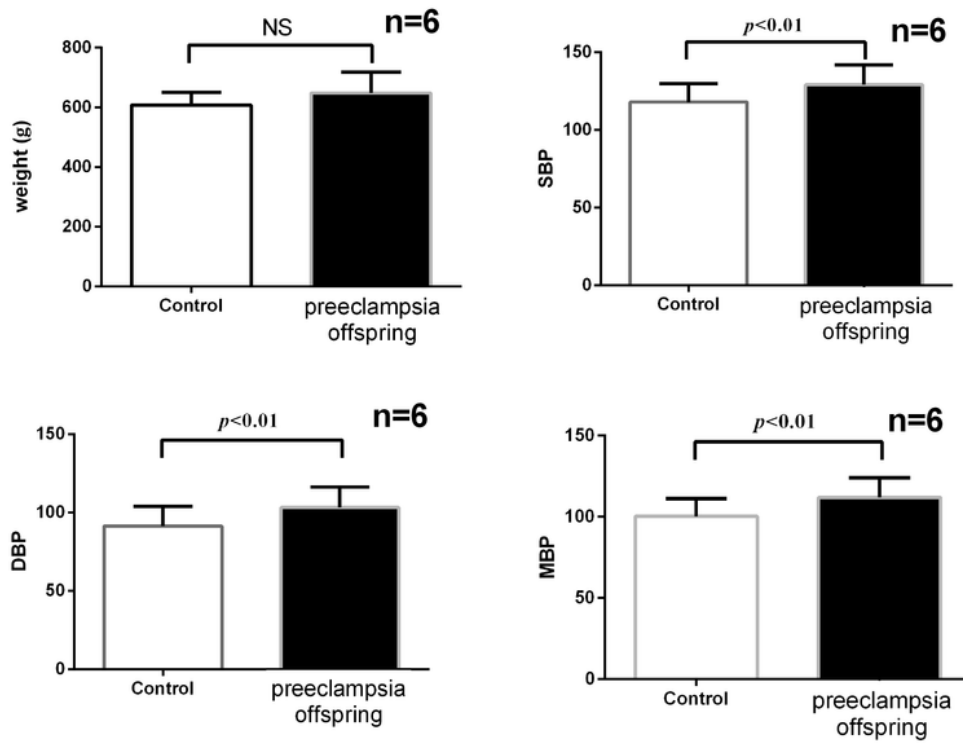
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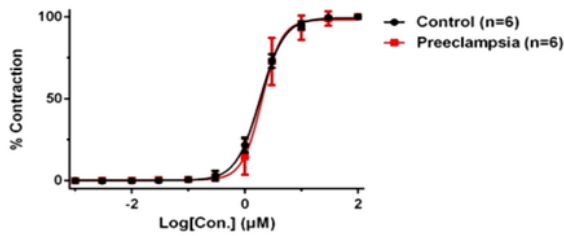
Figures

A



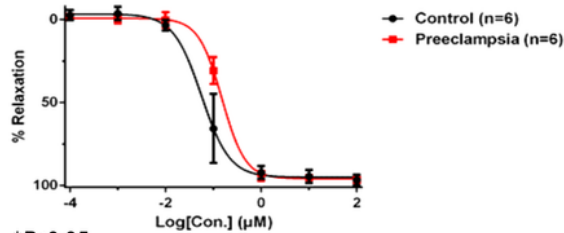
B

1 Year-old Offsprings Mesenteric Artery Contract Ability



	Control	Preeclampsia
EC50(μM)	1.854	1.999

1 Year-old Offsprings Mesenteric Artery Relax Ability



	Control	Preeclampsia
EC50(μM)	0.05627 *	0.1504

* $P < 0.05$

Figure 1

Rat model of preeclampsia. (A) blood pressure of offsprings from rat model of preeclampsia. (B) the logEC50 of mesenteric arteries of offsprings from rat model of preeclampsia.

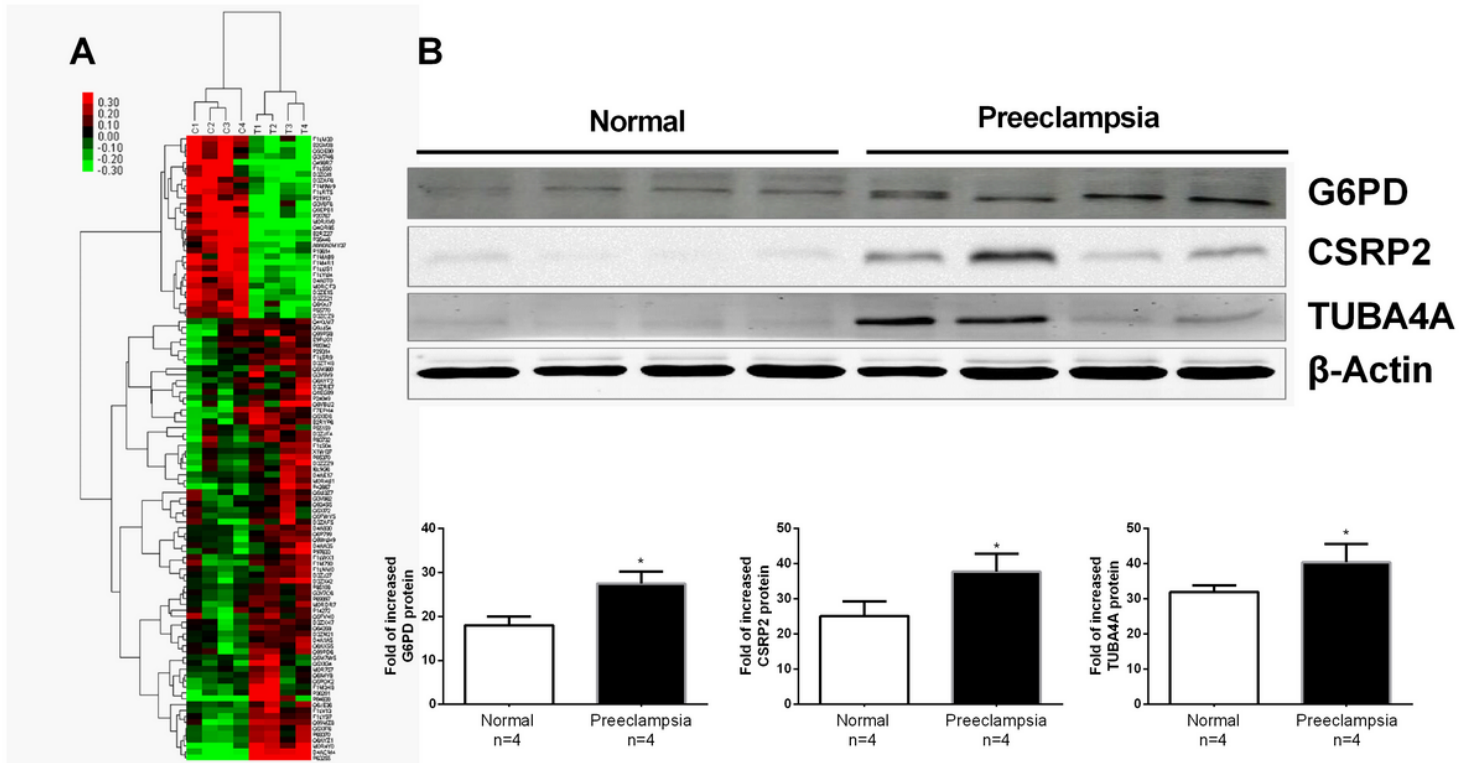


Figure 2

iTRAQ results of rat thoracic aortic. (A) Hierarchical clustering of DEPs in rat thoracic aortic. Black, deep green and bright red colors stands for no change, decreased, and increased, respectively. (B) Confirmation of different expression of three proteins. The relative expression levels of three proteins - G6PD, CSRP2 and TUBA4A - in rat thoracic aortic were confirmed with proteomic results. Group statistics are mean \pm SD (n=4). $p < 0.05$ versus controls.

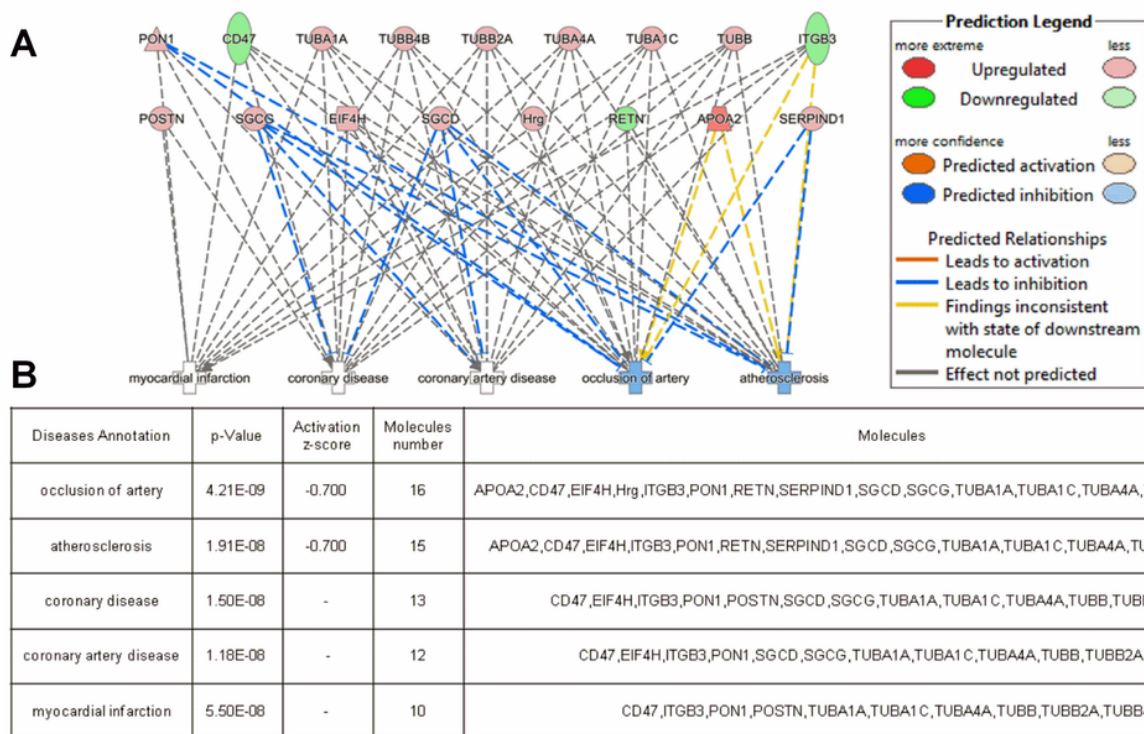


Figure 3

Relationship between specific DEPs with cardiovascular disease based on Ingenuity Pathway Analysis. Protein names and their temporal expression are outlined in the table. In this cardiovascular disease network, genes or gene products are represented using nodes, while correlation between two nodes is represented using an edge., with all edges supported by at least one publication, obtained from the Ingenuity Knowledge database. Red and green colors in the node denote upregulated and downregulated proteins, respectively. Interactions in the network as well as the relationships among molecules are summarized on the right side of the figure.

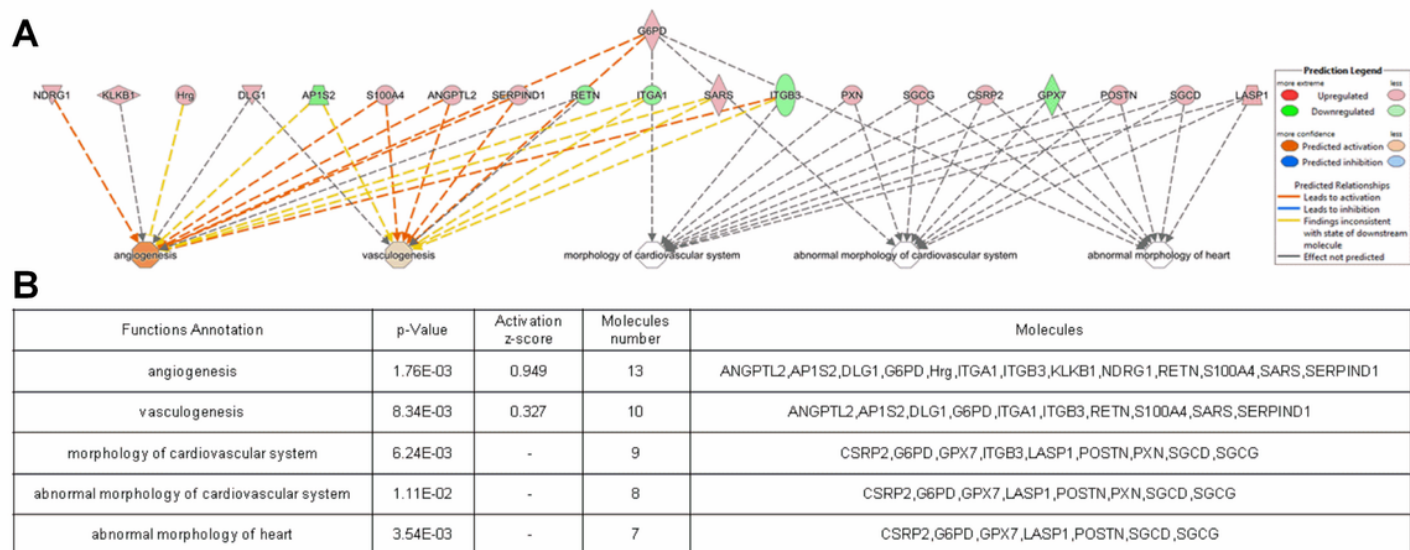


Figure 4

Profile of downstream effects of specific DEPs associated with cardiovascular function based on iterative analysis. Protein names and their temporal expression patterns are outlined in the table. Genes or gene products are represented using nodes, whereas the biological relationship between two nodes is indicated using an edge. All edges are supported by at least one publication as stored in the Ingenuity Knowledge database. Red and green colors in the node denote upregulated and downregulated proteins, respectively. Interactions in the network as well as the relationships among molecules are summarized on the right side of the figure.

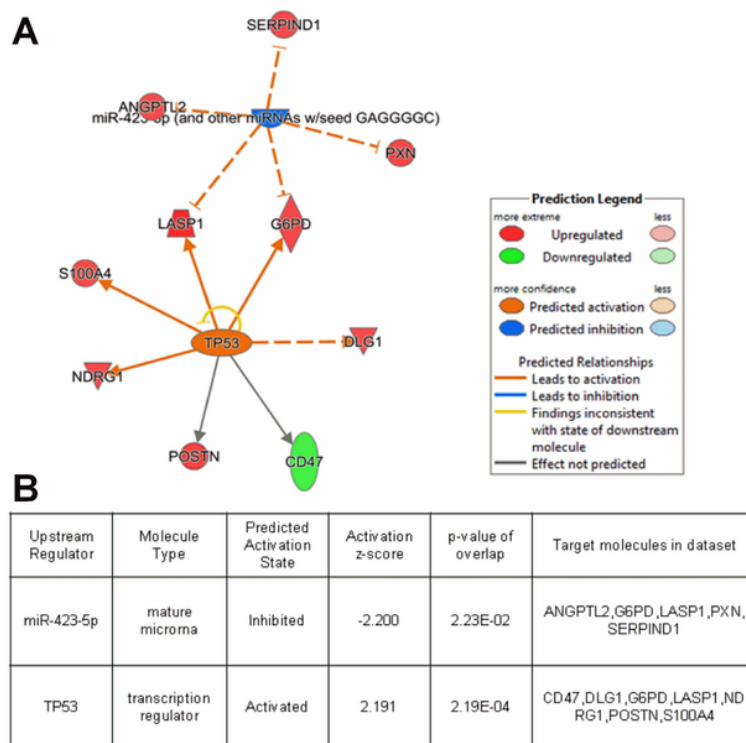


Figure 5

Upstream analysis by IPA based on 20 DEPs which were related with cardiovascular functions. Colorized nodes represented our input proteins. Red: up-regulated proteins. Green: down-regulated proteins.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalFigure1.tif](#)
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- [SupplementalTable1.xlsx](#)
- [SupplementalTable2.xlsx](#)