Gravida Serum POSTN and PAPP-A as the Potential Biomarkers for Fetal Congenital Heart Disease Based on Quantitative and Qualitative Proteomics

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Abstract

Background: Fetal congenital heart disease is the most common congenital defect worldwide. It has some missed diagnoses and a lack of disease biomarkers. We aim to seek objective biomarkers for noninvasive prenatal diagnosis of fetal congenital heart disease to reduce the missed diagnosis and explore its mechanism,

Methods: This study used data-independent acquisition and parallel reaction monitoring to explore potential protein biomarkers that co-exist in gravida serum and fetal amniotic fluid. Moreover, logistic regression and ROC curve to establish the diagnostic model of fetal congenital heart disease potential biomarkers and molecular biology experiments were performed to validate proteomics results and explore the mechanism.

Results: Proteomics and bioinformatics results show that 12 proteins co-exist in gravida serum and amniotic fluid. We identified POSTN and PAPP A in GS as candidate biomarkers and established the diagnostic model with a sensitivity of 100%, a specificity of 95% and the AUC value of 0.968 to diagnose congenital heart disease. In addition, the results of ELISA, IHC, and RT-PCR were consistent with those of proteomics. Moreover, POSTN may involve in fetal congenital heart disease through the TGF-beta signaling pathway.

Conclusion: It is the first time to find that POSTN and PAPP A in GS are related to fetal congenital heart disease, contributing to developing a novel noninvasive prenatal method to diagnose fetal congenital heart disease and reduce congenital disabilities.

Significance

In recent years, there are not enough objective biomarkers for the diagnosis of CHD, and the research on the pathogenesis of CHD mainly focuses on genes and hereditary disorders. Besides, abnormalities of extracellular matrix components are also involved in the pathological process of CHD, which is often ignored. For the first time, our study found that the expression level of extracellular matrix components POSTN and PAPP A in the peripheral blood of gravida with their CHD fetus is significantly different from that in normal fetal maternal peripheral blood. Alternatively, we can conclude that POSTN and PAPP A may be potential biomarkers for CHD. Our study is expected to lay a foundation for clinical prenatal noninvasive diagnosis of fetal CHD and the study about CHD mechanism.

1. Introduction

Congenital heart disease (CHD) is the most common congenital defect worldwide [1,2,3]. It accounts for one-third of all major congenital anomalies worldwide [4]. The ventricular septal and atrial septal defects are the most common CHD, with an incidence of about 5.29/1000, accounting for 19.6% for all CHD [2].

As we all know, prenatal diagnosis of CHD mainly depends on echocardiography. It is easily influenced by gestational week, fetal position, amniotic fluid (AF), and equipment, all of which can lead to missed diagnosis (20% ~ 30%). Moreover, fetal CHD was diagnosed by ultrasound already in the second trimester or later. Therefore, it is necessary to reduce the missed diagnosis and seek early diagnosis methods through objective biomarkers.

As we all know, AF is directly involved in fetal circulation, and the composition of AF can accurately reflect the development of the fetus. However, amniocentesis to obtain AF for diagnosing fetal diseases is invasive, which is somewhat risky for pregnant women and fetuses. While maternal blood intersects with fetal circulation through the placenta and umbilical cord, and maternal peripheral blood collection is safe and easy. Furthermore, proteins are the executors of life activities. So, we try to seek the protein biomarkers that co-exist in both AF and gravida serum (GS) to help prenatal diagnosis of fetal CHD.

Proteomics is a crucial method to discover biomarkers of diseases. Data-independent acquisition (DIA) collects data from a single sample and enables all peptides to be fragmented and analyzed in a given m/z window, enabling complete recording and highly reproducible quantification of all MS2 scans[5]. Parallel reaction monitoring (PRM) can monitor all products of the target peptide and screen out all other proteins and peptides in the sample to enhance the specificity and sensitivity of the quantification and validate one or several specific proteins. In our research, DIA was used to screen differentially expressed proteins in 30 samples of training sets (GS and AF from 9 cases of CHD group and 6 cases of the control group) and to verify these proteins in another 28 samples (GS and AF from 9 cases of CHD group and 5 cases of the control group) by PRM. Furthermore, these potential biomarker proteins were detected by ELISA in another 104 samples (GS and AF from 26 cases in the CHD group and 26 cases in the control group). Besides, we adopt immunohistochemistry (IHC) (4 cases in the CHD group and 5 cases in the control group) and RT-PCR (both 5 cases in the CHD group and the control group) with fetal myocardial tissue further to verify the expression differences of these potential biomarkers and try to find the signal path.

2. Material And Methods

2.1 Study design and inclusion criteria

Samples were all from the Department of Obstetrics and Gynecology, Wuhan University Zhongnan Hospital. The specific demographic data are shown in Table 1. The diagnostic criteria of CHD are detailed in the expert consensus [6]. This research program was approved by the ethics committee of Wuhan University Zhongnan Hospital (project number: 2020085, in supplement S2) and conducted in compliance with ethical guidelines of the Helsinki Declaration. The informed consent was informed and signed by all voluntary participants.

Table 1

Basic information characteristics of included patients

	CHD group	Control group	P value
DIA training sets(N=15)			
Maternal ageYear	27.50±3.02	30.50±5.32	0.352
Faternal ageYear	29.11±2.47	31.67±2.34	0.067
Gestational age(week)	24.69±5.82	21.00±3.36	0.147
NT (cm)	0.16±0.32	0.22±0.91	0.175
NIPT / Down's screening			0.221
Low-risk	9	5	-
High-risk	0	1	-
PRM validation sets(N=14)			
Maternal ageYear	29.12±4.58	29.00±3.95	0.948
Faternal ageYear	29.11±2.47	31.5±2.51	0.088
Gestational age(week)	24.84±5.76	22.37±2.08	0.491
NT(cm)	0.16±0.032	0.19±0.073	0.864
NIPT / Down's screening			0.63
Low-risk	9	0	-
High-risk	4	2	-
Elisa validation sets(N=52)			
Maternal ageYear	32±4.98	31.46±6.376	0.666
Faternal ageYear	30.58±5.278	31.5±7.355	0.213
Gestational age(week)	24.334±4.550	20.931±2.744	0.023
NT(cm)	0.149±0.073	0.174±0.071	0.158
NIPT / Down's screening			0.741
Low-risk	23	21	-
High-risk	1	3	-
Notes: N is the number of participants. Each participant had amniotic fluid and serum samples in this study. P<0.05 means statistically significant.			

Inclusion criteria for the CHD group: (1)fetal CHD was diagnosed by echocardiography; (2)The pregnant women naturally conceived were between 18 and 45 years old; (3) Couples are not close relatives. The inclusion criteria for the control group: (1)meet the above(2)(3); (2)The pregnant women were only older

(≥ 35) or the false positive of NIPT/screening for Down Syndrome or NT ≥ 0.25 cm, whose prenatal diagnosis results were normal. Exclusion criteria: (1) Fetus with extracardiac malformation; (2) Pregnant women have suffered from rheumatic heart disease, myocarditis, other non-structural heart diseases, hepatitis, abnormal renal and liver function, diabetes, or other acute or chronic infectious diseases.

Sample collection and preservation: (1) AF(2ml) were taken from pregnant women before amniocentesis, and corresponding maternal peripheral blood (2ml) was taken at the same time. AF supernatant and GS were taken out after centrifuge (4° C, 1000rpm/min, 10min) and then stored at - 80 ° C. (2) Fetal heart tissue: the heart tissue (2 ~ 4mm³) of induced labor fetus is taken. One part of the myocardial tissue was fixed in 4% formaldehyde for 48 hours before paraffin embedding, while the other was frozen in the refrigerator at - 80 ° C prepared for RT-PCR.

2.2 DIA

2.2.1 LC-MS / MS

GS was taken to remove the high peak protein (Thermo Fisher company, No:85165, kit column), so the total protein solution of AF can be obtained. All samples were enzymolized and desalted with an equal amount of protein. The peptides were redissolved with mobile phase A and then mixed evenly. Agilent 1100 HPLC system was used to separate the components in mobile phase pH=10. The peptide mixture was loaded by Agilent Zorbax Extend - C18 capture column (2.1× 150 mm, C18, 5 μm). The flow rate was 250nL/min, and the linear gradient was 63 min (0-10 min, 2%B; 10-10.1 min, 2-5%B; 10.1-37 min, 5-20%B; 37-48 min, 20-40%B; 48-48.01 min, 40-90%B; 48.01-58 min, 90%B; 58-58.01 min, 90-2%B; 58.01-63 min, 2%B; Mobile phase A=2%ACN and B=90%ACN). Mobile phase A and B ammonia was used to adjust pH to 10. Samples were collected for 8 to 50 minutes, one tube was collected every one minute in turn, and then the samples were collected in this order until the end of the gradient. A total of 10 components were obtained. After collecting, the samples were freeze-dried in a vacuum and drained. Before mass spectrometry detection, each sample and IRT standard (Biognosys, ThermoFisher) were mixed in the ratio of 1:10, and the coefficient of variation of IRT was less than 10%.

DDA mode database construction LC-MS/MS analyses are performed on a Q-extraction HF mass spectrometer (Thermo, USA). The peptide mixture was loaded by capillary C18 capture column. The analysis column was C18 reversed-phase column (ChromXP Eksigent). The flow rate was 300nL/min, and the linear gradient was 90 min. Full mass spectrometry scans were obtained in the mass range of 350-1650m/z with a mass resolution of 120,000 and an AGC target value of 3E6. All MS/MS spectra were collected using data-dependent high-energy collision pyrolysis in positive ion mode, and the collision energy was set to 27. MS/MS resolution was set as 30000, automatic gain control was set as 2E5, and the maximum injection time was 80 MS. The dynamic exclusion time was set to 40 s.

2.2.2 spectrum matching and database building

The machine signal is transformed into peptide and protein sequence information by matching the mass spectrum output with the theoretical spectrum generated by the FASTA library. Then the spectrum library is established by combining the sequence information, peptide retention time, and fragment ion information to facilitate the subsequent DIA analysis. Finally, the original LC-MS / MS files are imported into Spectronaut pulsar software to match and build the database. Database: UniProt-reviewed-Homo sapiens (Human)-20200817. The specific parameters of DDA and DIA are shown in supplement S4.

2.3 PRM

All samples were pre-scanned with the same amount of mix to correct the retention time of peptides. Based on the results of qualitative analysis, the identified target peptides were screened, and the trusted peptides were retained. PRM mode detection: The flow rate was 300nL/min, and the linear gradient was 90 min. MS/MS resolution was set as 30000 and an AGC target value of 1e5. Isolation Window 1.2m /z and the maximum injection time were 100 MS. The trusted peptides suitable for PRM analysis were introduced into the mass spectrometry software Xcalibur. After three times of PRM detection, the PRM original file data were PRM original file's data were analyzed by Skyline 3.7.0 software, and the target proteins and the target peptides were quantified.

2.4 Bioinformatics analysis

These identified proteins are annotated and analyzed(Including GO, pathway, PPI), which were based on the databases of UniProt(<https://www.uniprot.org/>), KEGG(<https://www.kegg.jp/kegg/pathway.html>), GO (<http://geneontology.org/>)and KOG / COG(http://cwmda.com/service_detail/90/122). At the same time, correlation analysis, expression pattern clustering heat map, Venn map were done for the difference comparison group data. Differential protein interaction analysis was performed by STRING (<https://www.string-db.org/>), and the correlation and interaction network was drawn by Cytoscape 3.6.1 software.

2.5 ELISA

POSTN (A102870-96T, Shanghai Fusheng Industrial Co., Ltd, China) and PAPPA (HM11110, Bioswap, China) in GS and AF were measured using commercially available ELISA kits. The experimental operation has been executed according to the corresponding instructions.

2.6 Immunohistochemistry (IHC)

IHC was performed on paraffin-embedded tissue sections. After being dewaxed, hydrated, repaired with EDTA buffer, and blocked with 5% BSA, tissues were incubated overnight at 4 ° C with primary antibodies to detect POSTN (ab219057, Abcam, USA). After incubation with HRP Goat Anti-Rabbit IgG (SAB4591, Bioswamp, China), slides were visualized by DAB-Substrate (Beyotime, China) and photographed by the Aperio ePathology Scanner (Leica, Germany). ImageJ software analyzed protein expression.

2.7 RNA isolation, cDNA synthesis, and RT-PCR

Total RNA was isolated by Invitrogen™ TRIzol™ Reagent (Thermo Fisher Scientific, USA), and cDNA was synthesized from the total RNA using PrimeScript™RT Master Mix (Takara, Japan). RT-PCR was performed on the Bio-Rad CFX96 (Bio-Rad Laboratories, USA) using SYBR Premix Ex Taq™ (Takara, Japan). The sequences of primers are in supplement S4. The relative expression of the mRNA was calculated by the $2^{-\Delta\Delta C_t}$.

2.8 Statistical analysis

Data analysis and the ROC curve analysis were processed by SPSS 22.0 (SPSS Inc, Chicago, IL). Metrological data were presented as means $\pm$ SD. An independent sample T-test was adopted when normal distribution and homogeneity of variance were met. Otherwise, Mann-Whitney U was performed. Fisher's exact test was utilized to compare the distribution of GO categories or KEGG pathways between the target protein set and the overall protein set and to perform enrichment analysis on the GO categories or KEGG pathways of the target protein set. $P < 0.05$ is considered statistically significant.

3. Results

3.1 The basic data

There was no significant difference between the two groups in age, NT, NIPT / Down screening results of patients in DIA, PRM, and ELISA sets. In ELISA sets, the gestational age in the CHD group was significantly older than that in the control group ($P < 0.05$). Because some control group samples have amniocentesis before the 4D Ultrasound examination (about 20~24week), while the CHD samples were often collected after that time.

3.2 Candidates differential protein from DIA

3.2.1 Trusted protein and Differential protein expression analysis

A total of 754 proteins and 6945 peptides were identified in serum, including 719 trusted proteins. 2007 proteins and 13101 peptides were identified in AF, including 1876 trusted proteins. The differential protein was screened based on trusted protein, according to foldchange = 1.2 times and p -value < 0.05 (T-test) between the two samples. 115 differential proteins (72 up-regulated and 43 down-regulated) were identified in serum, and 442 differential proteins (237 up-regulated and 205 down-regulated) were found in AF.

Among them, we focused on 27 differential proteins (Figure 1-A) that co-exist in serum and AF, and 19 proteins (16 proteins up-regulated, three proteins were down-regulated) showed a consistent expression trend. GO and KEGG enrichment analysis of these 19 proteins was performed according to the two FC mean values. Most proteins are enriched in bioadhesives and regulation, development process, extracellular matrix (or extracellular region), catalytic activity, enzyme regulatory activity (Figure 1-C and 1-D).

3.3 PRM validation of the protein expression

We selected 36 differential proteins (18 proteins co-exist in serum and AF, 13 only in AF, and 5 in serum) for PRM verification based on DIA results. There are 23 different proteins verified accurately (figure 2-A). Moreover, 12 proteins co-exist in serum and AF, including PAPP, were up-regulated, and POSTN was down-regulated ($P < 0.05$) (figure 2). These protein expression trends were consistent with DIA results, which indicated that our proteomic data were reliable.

3.4 Elisa validation and ROC analysis

We focused on PAPP and POSTN according to the results of DIA, PRM, and cardiovascular literature. To evaluate the expression level and efficiency of PAPP and POSTN in prenatal diagnosis of fetal CHD, 104 samples (GS and AF) were further verified by ELISA, and the multivariate logistic regression and ROC curve analysis were performed (Figure 3). Compared with the control, the level of PAPP in GS and AF of CHD group was significantly higher (0.835 ± 0.029 ng/ml VS 0.754 ± 0.033 ng/ml $P < 0.001$; 0.811 ± 0.017 ng/ml VS 0.721 ± 0.014 ng/ml, $P < 0.001$). The level of POSTN in GS and AF of the CHD group was lower than those in the control group (10.738 ± 8.799 ng/ml VS 17.033 ± 14.057 ng/ml, $P = 0.23$; 5.556 ± 1.487 VS 7.237 ± 2.194 ng/ml $P = 0.002$), although significantly down-regulated in CHD group only in AF samples. From a noninvasive point of view, we performed ROC curve analysis of PAPP and POSTN in GS instead of AF. The sensitivity of PAPP in GS was 90 %, and specificity was 0.95 %, and the area under the curve (AUC) values were 0.965($P = 0.000$, 95% CI: 0.913, 1.00). The sensitivity of POSTN in GS was 80%, and specificity was 55%, and AUC was 0.634($P = 0.148$, 95% CI: 0.904, 1.00). However, Combining PAPP with POSTN in GS, the sensitivity was 100%, the specificity was 95%, and the AUC was 0.968($P = 0.000$, 95% CI: 0.904, 1.00). Obviously, the diagnostic model of POSTN combined with PAPP in GS was more suitable for prenatal diagnosis of fetal CHD (Purple dotted line in Figure 3-B).

3.5 IHC and RT-PCR

IHC and RT-PCR were performed to analyze the differential expression of POSTN protein and the mRNA of TGF β signal pathway-related molecules between the CHD and control groups. IHC results showed that the expression of POSTN protein in the CHD group was significantly lower than that in the control group ($P < 0.05$) (Figure 4-A), which is consistent with the expression trend of POSTN in AF and GS. RT-PCR results showed that the relative expression of POSTN mRNA, TGF β 1 mRNA, Smad1 mRNA, Smad2 mRNA, and Smad3 mRNA were significantly lower than those in the control group ($P < 0.05$) (Figure 4-B). The primers involved in RT-PCR were shown in supplement 4.

4. Discussion

It is the first time to seek fetal CHD potential protein biomarkers co-exist in GS and AF by combining DIA with PRM. Compared with the control group, DIA, PRM, and ELISA results showed that POSTN in serum and AF of the CHD group was significantly down-regulated, while PAPPA was not significantly up-regulated. Similar results were also obtained from IHC and RT-PCR using fetal myocardial tissue. In addition, PPI results showed an interaction between POSTN and PAPPA (Figure 1-E). Studies also have shown that POSTN and PAPPA were related to cardiovascular development. Therefore, we did further research on POSTN and PAPPA as potential fetal CHD biomarkers.

POSTN(Periostin) is in the extracellular membrane, which involves cell-matrix interaction, cell fate determination, migration, and proliferation in development [7,8]. It is closely related to cardiovascular diseases, such as congenital heart valve disease, ventricular remodeling after myocardial infarction, myocardial fibrosis, etc. [8]. Therefore, it has been considered a new biomarker of cardiovascular diseases [9]. Although the periostin is expressed in early embryonic development, valves, outflow tract, atrioventricular tissues, and heart injury or disease [10-12], it is hardly expressed in healthy adult myocardium. Mesenchymal cell lines express periostin during heart development, promoting the formation of the atrioventricular outflow tract, valve, and chordate tendineae [11]. It is necessary for mesenchymal cells to transform into cardiac fibroblasts (CFS). Increased periostin expression was detected during embryonic development in developing atrioventricular tissues [12]. CFS chemotaxis is weakened in the absence of periostin, and the mesenchymal cells will transform into other mesoderm cells, like cardiomyocytes [13]. In our study, POSTN was significantly down-regulated in the CHD group, which may be related to the weakened ability of migration, differentiation, and proliferation in cardiac mesenchymal cells during cardiac development.

TGF- β / Smads signaling pathway is involved in cardiac development, and POSTN is the downstream response factor. That exogenous treatment of primary cardiac fibroblasts and vascular smooth muscle cells (VSMCs) with recombinant TGF- β 1 promoted the expression of Periostin via canonical SMAD-dependent signaling [14,15]. Periostin^{-/-} mice were susceptible to matrix stratification disorder, reduced TGF signal transduction, protein-polysaccharide aggregation, discontinuous valve lobules, and delamination defects [16]. Lack of Periostin can lead to low deposition and maturation of collagen fibers

in valve [17], atrial septal defect [13], abnormal structure of atrioventricular valve and supporting tensors, and abnormal differentiation of atrioventricular cushion stroma [13]. Furthermore, our RT-PCR results showed that the expression of TGF β 1-mRNA, Smad2/3 mRNA, POSTN mRNA in the CHD group was significantly lower than that in the control group, which suggested that POSTN may participate in the occurrence of CHD through the TGF- β / Smads signaling pathway and that the decreased expression of POSTN might result from inhibition of TGF- β /Smads signaling. However, some "gain or loss of function" experiments are necessary to prove it.

PAPPA, a secreted metalloproteinase, can specifically cleave IGFBP-4 (mainly) and IGFBP-5, then release the IGF. However, IGF (1/2) can promote the proliferation of mesoderm cells, thus promoting the formation of cardiac progenitor cells (differentiated into cardiomyocytes and vascular cells) [18]. PAPPA is concerned with pregnancy-related diseases and involves many cardiovascular diseases, considered a candidate marker of cardiovascular disease [19,20]. Interestingly, PAPPA also interacts with TGF- β signaling pathway downstream factors Smad2, Smad3, and Smad9 [21], consistent with our PPI results.

Our proteomic results showed an interaction between POSTN and PAPPA (figure1-E). Both POSTN and PAPPA were expressed in fibroblasts and mainly located in the extracellular matrix (ECM). The ECM contains many structural and non-structural proteins, interacting dynamically with unique and differentiating cell types which serve as a reservoir and processing site for signaling molecules and forming communication corridors for protein and genetic information. Therefore, the change of ECM components will disturb the signal transmission in the heart and lead to CHD. In our study, POSTN is significantly down-regulated, PAPPA is up-regulated, indicating that the dynamic balance and signal transduction may be disrupted. Eventually, abnormalities in cells' biological behavior result in abnormal cardiac structural development. In addition, PAPPA interacts with Smad2, Smad3, which may mean that PAPPA interacts with POSTN by TGF- β /Smads signaling pathway. Also, PAPPA is likely to serve as a critical molecular link between IGF and TGF- β signaling. However, this specific interaction mechanism needs further exploration.

By contrast, it avoids the bias of data-directed analysis (DDA) in the collection and fragmentation of high abundance peptides, DIA has significant advantages in the quantification of low abundance peptides. Furthermore, PRM can accurately quantify and replace the non-specific, low-throughput technologies based on immunoassay and obtain higher resolution and quantization accuracy. We combined DIA with PRM, bioinformatics analysis, and basic molecular biology techniques to seek out potential biomarkers of fetal CHD, such as POSTN and PAPPA.

Some advantages of this study are as follows: First, the potential biomarkers of fetal CHD co-exist in gravida serum and AF can genuinely reflect fetal development and be applied to clinic noninvasive prenatal diagnosis. Second, our results are representative and reliable. After screening the potential protein markers by DIA, PRM and ELISA were used to validate these candidate proteins in different sample sets. Furtherly, molecular biology technology was applied to verify these candidate proteins. Third, the related signal pathway and the potential mechanism of POSTN involved in fetal CHD were

explored initially. Nevertheless, this study has some shortcomings: it is challenging to collect large samples in a limited time. The sources of these potential biomarkers in AF and maternal serum need to be further explored microscopically. The specific mechanism of these biomarkers needs to be further explored through cell and animal experiments.

5. Conclusion

CHD is the most common congenital disability worldwide and lacks objective diagnostic biomarkers. However, we first found that POSTN and PAPP-A in maternal serum are related to fetal CHD, and there was an interaction between POSTN and PAPP-A, which the TGF β /Smads signaling pathway may mediate. The sensitivity and specificity of the combined diagnostic model of POSTN and PAPP-A in GS were 100% and 95%, respectively; the AUC value was 0.9684. Our results can contribute to developing new methods for noninvasive prenatal diagnosis of fetal CHD and may lay a foundation to reduce congenital disabilities and elucidate the mechanisms.

Abbreviations

CHD: Congenital heart disease; GS: gravida serum; AF: amniotic fluid; DIA: data-independent acquisition; PRM: parallel reaction monitoring; PPI: protein-protein interaction; POSTN: Periostin; PAPP-A: pregnancy-associated plasma protein A; TGF- β : transforming growth factor- β ; Smad2/3: Mothers against decapentaplegic homolog 2/3. LC-MS/MS: Liquid chromatography-tandem mass spectrometry; FDR: False discovery rate; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PCA: Principal component analysis;

Declarations

Acknowledgments

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Author contributions

Yi Xia: Sample collection; Methodology; Data processing and analysis; Writing-original draft; Writing-original draft. Lin Cheng: Sample collection (supporting); Jie Duan: Prenatal diagnosis (equal). Jian-hong Ma(equal): Prenatal diagnosis (equal). Dan lu: Prenatal diagnosis (equal). Juan Liu: Sample collection

(supporting). Yuan-Zhen Zhang: Project design and guidance; Funding support; Writing-review and revise; Resources; Prenatal diagnosis (equal).

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Ethics approval and consent to participate

This research protocol was approved by the ethics committee of Wuhan University Zhongnan Hospital (project number: 2020085, in supplement S2) and adhered to the Helsinki Declaration's ethical guidelines. The informed consent was informed and signed by all voluntary participants.

Data availability statement

The mass spectrometry proteomics data [PXD026855] has been deposited to the proteomics database (<https://www.iprox.cn/>), and the temporary access is provided in appendix 5. Moreover, other data that support this study's results are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declared that they have no conflicts of interest in this work.

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Figures

the dot represents the connection degree, and the larger the dot indicates the higher the connection degree.

2-A

2-B

2-C

Figure 2

The results from PRM. 2-A shows PRM identified the differential proteins. 2-B and C show the box plot of PAPP A and POSTN, respectively. The x-axis and Y-axis, respectively, show the sample grouping and the

protein expression value intensity. * $p < 0.05$, ** $p < 0.005$, and NS mean no significant difference.

3-A-1

3-A-2

3-B

Figure 3

The average level POSTN and PAPP from Elisa and the regression and ROC analysis. 3-A shows the average level of POSTN and PAPP in serum and AF(* $p < 0.05$ ** $p < 0.01$). The prenatal diagnosis of

fetal CHD model with PAPPA combined with POSTN in GS was shown in the purple dotted line in figure 3-
B (sensitivity 100%, specificity 95%, AUC 0.968, cutoff value 0.5156736, $P < 0.05$, 95% CI: 0.904, 1.00).

Figure 4

The results from IHC and RT-PCR. 4-A shows the IHC analysis of POSTN in fetal myocardial tissue (magnification x200) of CHD group (4-A-1, N=4) and control group (4-A-2, N=5). 4-B shows relative mRNA

expression of POSTN, TGF β 1, Smad2, and Smad3 in the fetal heart. * $P < 0.05$, * * $P < 0.01$, *** $P < 0.001$.

Supplementary Files

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