Original Research

Maternal serum-derived exosomal lactoferrin as a marker in detecting and predicting ventricular septal defect in fetuses

Suping Li[1,2], Yuxia Jin[2], Ping Tang[2], Xiaodan Liu[2], Xiaojun Chai[2], Jinhua Dong[2], Xuan Che[2], Qinqin Zhou[2], Meidi Ni[2] and Fan Jin[1]

[1]Department of Reproductive Endocrinology, Women's Hospital, Zhejiang University School of Medicine, Hangzhou 310006, China; [2]Department of Fetal Medicine Center, Jiaxing Maternity and Child Health Care Hospital, Jiaxing University Affiliated Women and Children Hospital, Jiaxing 314050, China

Corresponding author: Fan Jin. Email: jinfan@zju.edu.cn

*SL and YJ contributed to this study equally.

Impact statement

Congenital heart disease is the most frequent congenital malformation. Fetal ultrasound examinations are frequently used to detect fetal congenital heart disease after gestational age of 24–28 weeks. However, this delay in detection and diagnosis often causes great physical and mental harm to pregnant women and their families. In the study, we discovered a lower level of lactoferrin protein in the exosomes from ventricular septal defects than that from healthy fetuses, which may serve as a potential biomarker for non-invasive prenatal diagnosis of fetal ventricular septal defects in the first trimester of pregnancy. Our research provides new insight for pathogenesis of congenital heart disease and might have implications in the early intervention, as well as treatment of congenital heart malformations.

Abstract

Among different types of congenital heart diseases, ventricular septal defect is the most frequently diagnosed type and is frequently missed in early prenatal screening programs. Herein, we explored the role of maternal serum-derived exosomes in detecting and predicting ventricular septal defect in fetuses in the early stage of pregnancy. A total of 104 pregnant women consisting of 52 ventricular septal defect cases and 52 healthy controls were recruited. TMT/iTRAQ proteomic analysis uncovered 15 maternal serum exosomal proteins, which showed differential expression between ventricular septal defect and control groups. Among these, four down-regulated proteins, lactoferrin, SBSN, DCD, and MBD3, were validated by Western blot. The protein lactoferrin was additionally verified by ELISA which was able to distinguish ventricular septal defects from controls with area under the ROC curve (AUC) 0.804 ($p < 0.001$). Our findings reveal that lactoferrin in maternal serum-derived exosomes may be a potential biomarker for non-invasive prenatal diagnosis of fetal ventricular septal defects.

Keywords: Ventricular septal defect, exosomes, biomarker, prenatal diagnosis, congenital heart disease

***Experimental Biology and Medicine* 2022; 247: 488–497. DOI: 10.1177/15353702211060517**

Introduction

Congenital heart disease (CHD) is a common congenital malformation, affecting approximately 1% of live births worldwide.[1–3] CHD also remains as the primary cause of mortality from birth defects under the age of 5 in both developed and developing countries.[1,2] Ventricular septal defects (VSDs) are the most frequent type of CHD and account for approximately 32% of all heart malformations diagnosed during the first year of life.[4] VSDs may arise from multifactorial causes and are frequently missed in prenatal screening.[5] Consequently, there remains a clinical need for non-invasive biomarkers that can predict VSD occurrence, and to identify novel prevention strategies to reduce the incidence of VSDs.

Exosomes are single-membrane, nano-sized vesicles (30–200 nm in diameter) secreted by nearly all body cells into the extracellular environments.[6] Exosomes harbor a wide range of signaling biomolecules (e.g. proteins, lipids, nucleic acids, and glycoconjugates)[7] and take part in cell-to-cell communication. Numerous studies have demonstrated important roles of exosomes in diverse physiological processes, as well as disease pathogeneses, including development, tissue homeostasis, immunity, cancer, and neurodegenerative disease.[8]

Recently, the discovery of exosomal proteins or miRNAs in maternal circulation has paved new avenues for early detection of pregnancy-linked disorders, for instance preterm delivery,[9] preeclampsia,[10] fetal brain development,[11] and fetal cardiac development deficiency.[12] Previous investigations have demonstrated that the placenta releases specific exosomes into maternal circulation and the amount of exosomes in maternal circulation rises remarkably with the advancement of the pregnancy (from 6 to 12 weeks).[13] However, no studies have explored a potential role of circulating exosomal proteins as prenatal predictive signatures of birth defects.

Herein, we explored the overall alterations in protein expression patterns in serum-originated exosomes from expectant women carrying fetuses with VSDs using TMT protein profiling assessment and Western blot. The prospective protein markers were verified again in the early exosomes from pregnant women (11–14 weeks) by ELISA. The present results broaden the utility scope of exosomal proteins as possible biomarkers of congenital heart disease, in particular, ventricular septal defects subtypes.

Materials and methods

Study population and sample collection protocols

Serum samples were collected from VSD-fetus pregnancies (11–14 weeks, n = 52) (abbreviation: VSDs) and healthy-fetus pregnancies (11–14weeks, n = 52) (abbreviation: Ctr) after conventional serological screening for Down syndrome from June 2018 to June 2020. We conducted a retrospective study on VSDs (24–28 weeks) diagnosed prenatally with ultrasound in the Jiaxing University Affiliated Maternity and Child Health Care Hospital. VSDs inclusion criteria: singleton pregnancy and pregnant women whose fetus was diagnosed with congenital ventricular septal defect by prenatal echocardiography without other malformations. Exclusion criteria: (1) pregnant women with pregnancy complications; (2) pregnant women diagnosed with chromosomal abnormalities in the fetus; (3) pregnant women diagnosed with multiple malformations in the fetus. Inclusion criteria also for the control group: pregnant women carrying normal fetuses admitted to our hospital. The fetuses in the control group should be free of diagnosis of heart malformations or any other deformities following neonatal ultrasound examination after birth. The control group matched to VCD group in pregnant women's age, gestational weeks, and the time of blood sampling. The demographic along with the clinical features of this study are documented in Table 1 (More detailed information about participants in supplementary Table 1) . The study design included three phases and detailed flow diagram of the study analysis design is shown in Figure 1.

The serum sample collection was approved by the Scientific and Ethical Committee of Jiaxing University Affiliated Women and Children Hospital. All samples were received with a written informed consent granted by the subjects. Besides, we adhered to the Declaration of Helsinki in conducting the study.

Table 1. Clinical features of pregnant women for VSD and fetuses used in TMT/iTRAQ, Western blotting, and ELISA.

	TMT/iTRAQ		
Characteristic	VSDs (n = 6)	Ctrl (n = 6)	p
Gestational age (weeks)	12.3 ± 0.1	12.4 ± 0.1	0.72
Maternal age (years)	27.7 ± 2.8	28.0 ± 2.3	0.83
	Western blotting		
Characteristic	VSDs (n = 10)	Ctrl (n = 10)	p
Gestational age (weeks)	12.3 ± 0.7	12.4 ± 0.7	0.86
Maternal age (years)	30.7 ± 4.6	30.7 ± 3.9	1
	ELISA		
Characteristic	VSDs (n = 36)	Ctrl (n = 36)	p
Gestational age (weeks)	12.5 ± 0.5	12.5 ± 0.5	0.88
Maternal age (years)	28.9 ± 4.3	28.8 ± 3.8	0.93

Exosome isolation

The serum exosomes were extracted via the ExoQuick precipitation approach (System Bioscience, Inc., SBI, Mountain View), as described by the manufacturer. In brief, spinning of the serum samples was done at 3000*g* for 15 min to eliminate the cells along with the cell debris. Then serum supernatant (250 μl) was mixed with ExoQuick Exosome Precipitation Solution (63 μl) (4:1), followed by refrigeration of the mixture was for 30 min. After refrigeration, the mixture was spun** for 30 min at 1500*g*, and the supernatant was aspirated. Afterwards, spinning of the residual solution for 5 min at 1500*g* was done and removal of all traces of fluid conducted via spiration. The exosome pellet was resuspended in 100 μl of PBS and kept at –80°C.

Protein extraction and trypsin digestion

Sonication of each exosome sample (in lysis buffer consisting of 8 M urea, 1% and Protease Inhibitor Cocktail) was done thrice on ice with a high intensity ultrasonic processor (Scientz). Removal of the remaining debris was done via spinning for 10 min at 12,000*g* at 4°C. Thereafter, we collected the supernatant, followed by quantitation of the proteins with the BCA kit as documented by the manufacturer. After that, the samples were inoculated with 5 mM dithiothreitol for digestion at 56°C for 30 min, and subsequent alkylation for 15 min was done in darkness using 11 Mm iodoacetamide at room temperature. Next, 100 mM TEAB was introduced to urea in a level of less than 2 M to dilute the proteins. Lastly, we introduced trypsin at a 1:50 trypsin-to-protein mass ratio in the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second digestion for four hours.

TMT/iTRAQ labeling and HPLC fractionation

Following the trypsin digestion, desalting of peptides was done via Strata X C18 SPE column (Phenomenex), followed by vacuum drying. Reconstitution of peptides in 0.5 M TEAB was performed, and processing was conducted as described in the manual of the TMT kit/iTRAQ kit.

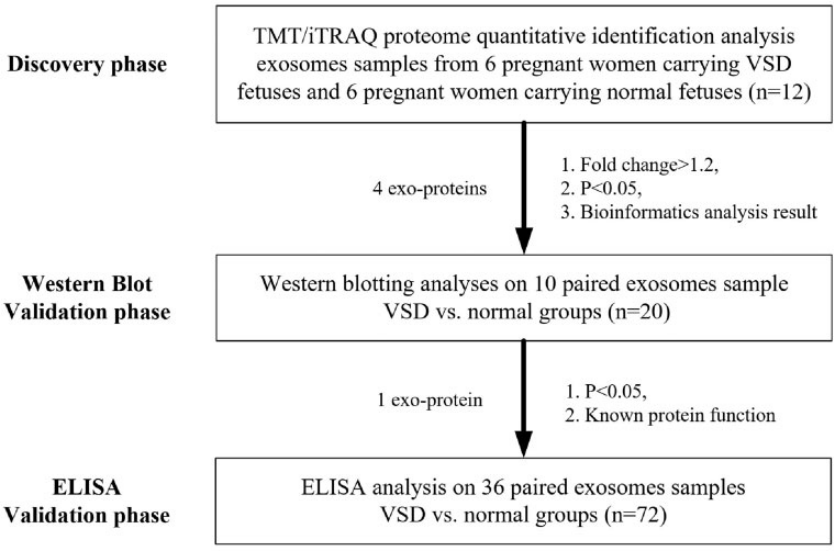

Figure 1. Study flow diagram of the analysis design. Subjects from three different populations were divided into discovery, Western Blot validation, and ELISA validation phases of the study.

Concisely, we thawed a TMT/iTRAQ unit followed by reconstitution in acetonitrile. Thereafter, we incubated the peptides at room temperature for 2 h and subsequently pooled. Next, desalting and drying were done via vacuum spinning. Fractionation of the tryptic peptides was performed via high pH reverse-phase HPLC with a Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). In brief, we first treated the peptides with an 8% to 32% gradient of acetonitrile (pH 9.0) over 60 min into 60 fractions. Thereafter, combination of the peptides was done into eight fractions, followed by drying through vacuum centrifugation.

LC-MS/MS analysis

Dispersion of the tryptic peptides was done in solvent A (formic acid; 0.1%), and subsequent direct loading done onto a home-designed reverse-phase analytical column (75 μm i.d, 15-cm length). The gradient consisted on an increment beginning at 6% up to 23% of formic acid (0.1%) in acetonitrile (98%) (solvent B) for over 26 min, 23% to 35% for 8 min and climbing to 80% in 3 min, and finally holding at 80% for the last 3 min, all at a constant rate of flow of 400 nL/minute on an EASY-nLC 1000 UPLC platform.

Subjection of the peptides to the NSI source was done, and then tandem mass spectrometry (MS/MS) was done in an Orbitrap Fusion^{TM}Tribrid^{TM} (Thermo) linked online to the UPLC. Detection of intact peptides was performed with the Orbitrap (60,000× resolution). An NCE setting of 35 was employed to choose the peptides for MS/MS; detection of ion fragments was conducted on the Orbit rap (at 15,000× resolution). We applied a data-dependent process alternating between one MS scan and a subsequent 20 MS/MS scans on the top 20 precursor ions above a cutoff intensity of more than 5E3 in the MS survey scan with 15.0 s exclusion dynamic. A 2.0 kV electrospray voltage was applied. Automatic gain control (AGC) was utilized to evade overfilling of the orbitrap; 5E4 ions were accumulated for generating MS/MS spectra. In the MS scans, the m/z scan range was 350 to1550. We set fixed first mass at 100 m/z.

Bioinformatic analysis

These exo-proteins that were remarkably differentially expressed ($p < 0.05$) were inspected further, and we retained those that exhibited a differential expression ratio of over 1.2 for GO term analysis. The ontology constitutes three components: biological processes (BP), cellular component (CC) as well as molecular function (MM). The log10 (p-value) designates an enrichment score reflecting GO term enrichment significance among the proteins that are differentially expressed. Identification of cascade is based on the molecular cross-talk with reaction networks performed via KEGG pathway analysis. A log10(p-value) via Fisher's Exact Test defined the enrichment scores. The top 10 terms of functional cascade assessment are presented this study.

Western blotting

Exosomes dispersed in PBS were lysed with the RIPA buffer. Total exosomal protein levels were measured with the Pierce BCA Protein Assay Kit. The lysates were centrifuged, and the supernatant protein concentration was quantified. Fractionation of proteins was done on an SDS-PAGE gel an d transfer-embedded onto PVDF membranes. After that, blocking of the membranes was done using 5% dry milk in TBS with 0.05% Tween for 1 h. The blots were inoculated with exosome-distinct antibodies (CD81, CD63, and CD9, 1:10 00; Abcam), and anti-placenta alkaline phosphatase (PLAP; 1:1000; Abcam) LTF (1:500;Abcam), MBD3(1:2000; Abcam), DCD (1:1000; Abcam), SBSN (1:500; Abcam), PR3 (1:500; Abcam), and β-actin (1:1500; Santa Cruz) antibodies overnight at 4°C. T hen the blots were inoculated with goat anti-mouse-rabbit pierce antibody (1:500 0; Thermo). The immune-reactive

bands were tested with Super Signal West Duration Substrate as described by the manufacturer and visualization done with the Fluor Chem E imaging machine (Protein Simple, San Jose, USA).

Transmission electron microscopy

Twenty microliters of exosome suspension were applied onto the electron mirror copper mesh grid, and negative staining (in 2% uranyl acetate aqueous solution) was done for 1 min. Blotting of the excess sample was done using a filter paper from the grids and dried at room temperature. The samples were assessed on a field emission transmission electron microscope at 80 kV (Tecnai G2 SpiritBio TWIN) and linked to a direct electron detector (Falcon | FEI Company).

Nanoparticle tracking analysis

A Nano Sight NS300 instrument (Malvern Panalytical, UK) was employed to assess the exosome size distribution, with a 488 nm laser, as well as sCMOS camera module (Malvern Panalytical, UK) as described in the manufacturer's manual. Prior to analysis, we diluted the samples with PBS at 1:1000 to yield a particle distribution of between 10 and 100 particles/image, and then a Nanoparticle Tracking Analysis Platform was adopted to assess the particles.

ELISA

ELISA kits were employed to assess the quantities of exo-originated LTF (CSB-E0883, Abcam, USA) in serum, as per the manual of the manufacturer. Concisely, the RIPA buffer was utilized to lyse the exosomes for 30 min at 4°C and planted in a 96-well plate, and then inoculated with the antibody mix and incubated.

Afterwards, the unbound antibodies were rinsed off. Next, the plates were inoculated with the TMB substrate to form a blue coloration. Upon addition of Stop Solution, the OD was measured at 450 nm. Standard curve obtained from the known levels of the respective recombinant proteins was employed to compute the protein concentrations.

Statistics

All data were given as mean ± standard deviation (SD), and analysis was implemented in SPSS 23.0 software (SPSS Inc., Chicago, IL). Comparisons between two group comparisons were determined using parametric analyses and unpaired Student's t-test. The multiple group comparisons were determined using one-way ANOVA followed by the Fisher least significant difference method. $p < .05$ denoted statistical significance.

Results

Isolation and characterization of serum exosomes

Table 1 presents the clinical features of the study groups. Transmission electron microscopy (TEM) of the harvested exosomes revealed circular membrane-bound structures exhibiting sizes varying from 30 to 150 nm in diameter, which was congruent with previously documented features of exosomes (Figure 2(a)).[10] The size and concentration of Vsd-exo and Ctr-exo were determined via nanoparticle tracking analysis (NTA) (Figure 2(b)). Western blotting of the isolated exosomes detected the frequently reported exosomal tetraspanin markers CD63, CD81, and CD9, and the placenta-specific exosome marker PLAP (Figure 2(c)).

Serum exo-protein identification and assessment of relative abundance

To search for VSD-related serum exo-proteins, total exosome proteome was profiled using a TMT-labeling proteomics approach. Overall, we determined 312 proteins and 272 proteins were quantified, of which 15 proteins expressed differentially, including nine down-regulated and six up-regulated proteins (Table 2) (The quality control results of exosomal protein in Supplementary Figure 1).

Bioinformatics analysis result of differentially expressed exo-proteins

We carried out GO along with KEGG pathway assessment for these differentially expressed exo-proteins. On the basis of the enrichment score (–log10(p-value)), the GO processes were determined for these up-regulated, as well as down-regulated proteins (Figure 3(a) and (b)) (Cluster analysis heat map results in Supplementary Figure 2 and Figure 3). For up-regulated proteins, the most abundant GO terms consisted of immune response-activating cell surface receptor signaling cascade and immune response-modulating cell surface receptor signaling cascade in BP; antigen binding in MF; antigen binding in CC. The top GO process forecasted by down-regulated proteins were antimicrobial humoral response, response to fungus and positive modulation of toll-like receptor signaling cascade in BP; nucleic acid binding in MF; azurophil granule lumen in CC. Our data exhibited that the up-regulated along with the down-regulated proteins were linked to developmental processes in the heart. However, our KEGG pathway analysis did not identify the relevant signal pathway. These data indicate that the involved cascades control fetus heart development.

Identification of protein specific to VSD (Western blot validation)

After the TMT analysis, we carried out Western blotting to validate the key protein expression differences discovered by TMT analysis. We selected LTF, SBSN, DCD, and MBD3 for further verification, as these proteins are known to have important functions that may regulate heart development. As shown in Figure 4(a) and (b), the Western blot results confirmed that all four proteins were differentially expressed, in agreement with the TMT results.

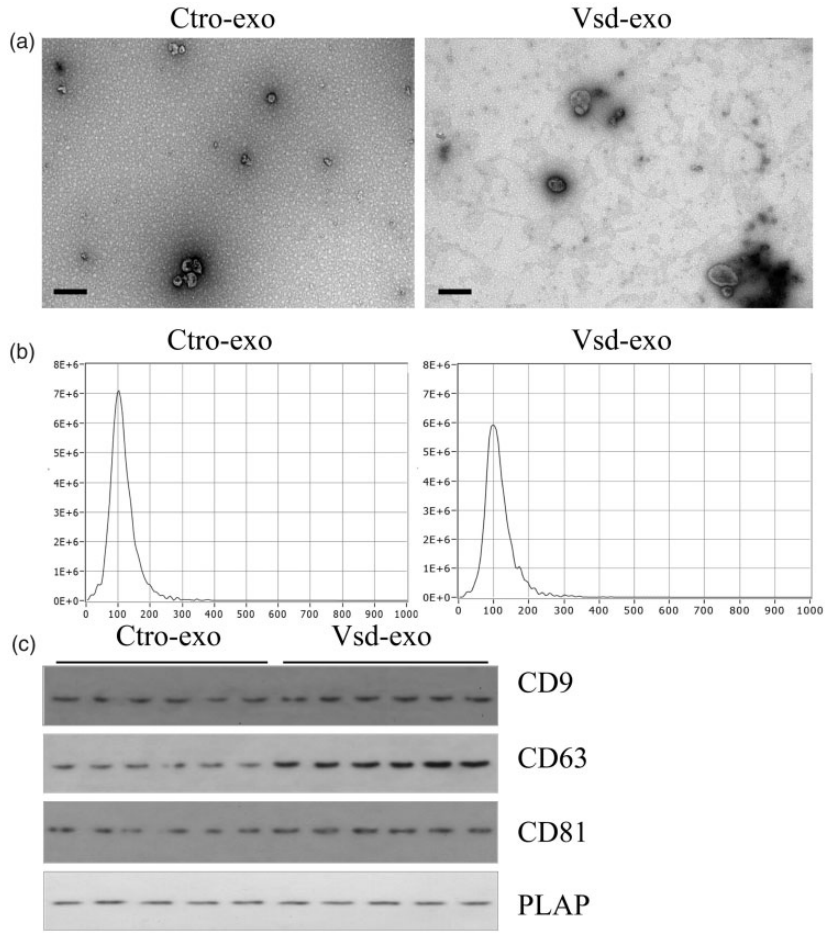

Figure 2. Assessment of exosomes in maternal circulation. (a) Transmission electron micrographs illustrating exosomes from healthy pregnant woman (Ctr-exo) and exosomes from pregnant woman with fetus ventricular septal defect (Vsd-exo; n = 6 per group) (scale bar = 200nm). (b) Profiles illustrating Nanoparticle Tracking Analysis (NTA) of Ctr-exo and Vsd-exo (n = 6 per group). The mean diameter of particles was approximately 100 nm. C.CD9, CD63, and CD81 (common exosomes enriched markers) (n = 6 per group) and PLAP immunoblots of Ctr-exo and Vsd-exo (n = 5 per group).

Table 2. The fold change >1.2 down-regulated and up-regulated proteins identified from iTRAQ analysis.

Protein accession	Gene name	Protein description	Regulated type	MW (kDa)	P/C p value	P/C ratio
P24158	PRTN3	Myeloblastin OS = Homo sapiens GN = PRTN3	Down	27.807	0.0179	0.24
Q2KHM9	KIAA0753	Protein moonraker OS = Homo sapiens GN = KIAA0753	Down	109.41	0.0304	0.34
P59666	DEFA3	Neutrophil defensin 3 OS = Homo sapiens GN = DEFA3	Down	10.245	0.00608	0.34
A0M8Q6	IGLC7	Ig lambda-7 chain C region OS = Homo sapiens GN = IGLC7	Down	11.303	0.0174	0.4
P81605	DCD	Dermcidin OS = Homo sapiens GN = DCD	Down	11.284	0.0051	0.44
P02788	LTF	Lactotransferrin OS = Homo sapiens GN = LTF	Down	78.181	0.0005	0.46
Q6UWP8	SBSN	Suprabasin OS = Homo sapiens GN = SBSN	Down	60.54	0.00636	0.57
A6NJ08	MBD3L5	Putative methyl-CpG-binding domain protein 3-like 5 OS = Homo sapiens GN = MBD3L5	Down	22.976	0.00944	0.65
P18428	LBP	Lipopolysaccharide-binding protein OS = Homo sapiens GN = LBP	Down	53.383	0.0164	0.78
P01714	IGLV3-19	Immunoglobulin lambda variable 3-19 OS = Homo sapiens GN = IGLV3-19	Up	12.042	0.0499	1.24
P01602	IGKV1-5	Immunoglobulin kappa variable 1-5 OS = Homo sapiens GN = IGKV1-5	Up	12.781	0.0153	1.3

(continued)

Table 2. Continued.

Protein accession	Gene name	Protein description	Regulated type	MW (kDa)	P/C p value	P/C ratio
A0A0C4DH31	IGHV1-18	Immunoglobulin heavy variable 1-18 OS = Homo sapiens GN = IGHV1-18	Up	12.82	0.0149	1.35
P01624	IGKV3-15	Immunoglobulin kappa variable 3-15 OS = Homo sapiens GN = IGKV3-15	Up	12.496	0.00386	1.37
A0A0C4DH29	IGHV1-3	Immunoglobulin heavy variable 1-3 OS = Homo sapiens GN = IGHV1-3	Up	13.008	0.0476	1.38
P01742	IGHV1-69	Immunoglobulin heavy variable 1-69 OS = Homo sapiens GN = IGHV1-69	Up	12.659	0.0286	1.63

Identification of protein specific to VSD (ELISA validation)

After the Western blot validation, we also performed ELISA analysis to further explore the effectiveness of the selected exosomal protein LTF in predicting fetal ventricular septal defect. We conducted ELISA verification experiments in 36 VSDs and 36 controls. As shown in Figure 5, protein LTF was significantly down-regulated in the serum of VSD mothers. Exosomal protein LTF had a high diagnostic accuracy with an AUC of 0.804 (under this threshold, sensitive value is 0.628889, specific value is 0.805556) (Figure 6). Together, these results indicate that maternal serum exoprotein LTF can differentiate between VSDs and healthy controls.

Discussion

The prenatal diagnosis of fetal congenital heart disease could usually only be made after the gestational age of 24–28 weeks by fetal ultrasound examination, which often causes great physical and mental harm to pregnant women and their families. Specific molecular signatures for prenatal diagnosis of congenital heart malformations are lacking. Herein, we uncovered a potential role of maternal serum exosomal protein LTF in early diagnosis of fetal ventricular septal defect. So far, this is the first study detecting the expression levels of circulating proteins in the serum exosome from pregnant women carrying fetuses with CHDs and may have implications in the early prevention and treatment of fetal congenital heart diseases.

The functions of exosomes in maternal–fetal communication are only beginning to be elucidated. Interestingly, the placenta-derived exosomes have been found in maternal plasma as early as six weeks of gestation age, and they continuously increase over the course of the first trimester of pregnancy.[13] A recent investigation has documented that exosomes also regulate pivotal processes during pregnancy, consisting of the immune responses, migration/infiltration of placental cells, as well as cellular adaptations to the pregnancy-associated physiological changes.[14] Our study identified a total of 15 proteins, which showed differential expression between serum of the fetus with VSD and healthy fetuses. Among them, nine were down-regulated and six were up-regulated. According to bioinformatics analysis results, we selected four exosomal proteins most likely to be associated with fetal VSD to be verified by Western blotting. We finally selected one of the candidates, LTF, as a potential biomarker after case–control WB experiments (n = 10). ELISA experiments showed that maternal peripheral blood serum exosomes LTF levels can be used to distinguish VSD from controls.

In recent years, exosomes have been considered as communication tools between cells. Many studies have shown that the contents of exosomes undergo subtle changes before the disease occurs.[13,15] It is exciting that exosomes are membrane-encapsulated nano- or microparticles, with their internal contents well protected from external proteases and other enzymes.[16] These characteristics make exosomes an ideal stable reservoir for promising circulating signatures for some diseases. Investigations have documented that plasma-originated extracellular vesicles harbor prognostic biomarkers, as well as possible treatment targets for myocardial ischemic injury,[17] and Shi *et al*. have illustrated that maternal exosomal miRNA in diabetes could cross the maternal–fetal barrier and participate in the cardiac development deficiency.[12] In our current study, we found that serum exosomal protein LTF levels in fetal VSD pregnant women are significantly lower than healthy fetal pregnant mothers. Thus, we suggest that exosomes contain key protein components that may vary according to pathological or physiological environments and are capable of affecting the development of the fetus. Our results will support the design of predictive models of abnormal fetal heart developments and give a good opportunity for early intervention and treatment.

GO along with the KEGG cascade analyses were conducted to explore the biological functions, as well as molecular mechanisms of associated exo-proteins in the progress of VSDs. The data illustrated that the dysregulated exoproteins could be involved in the development during VSD progression. Regarding GO pathway analysis, the toll-like receptor 4 signaling cascade can activate important cardio-protective signaling pathways including ERK1/2 and/or PI3K/AKT.[18] More specifically, LTF could trigger the biosynthesis of vitamin B6 and protect cardiovascular function via activating the ERK1/2 and PI3K/AKT cascade.[19] LTF, a glycoprotein, participates in numerous biological processes, such as antioxidant, anti-inflammatory, and anti-cancer activities.[20] Results obtained by other investigations have documented a negative impact of inflammation factors on fetal development.[21] Therefore, we speculate that the content of LTF in maternal serum exosomes reaches a certain level to maintain effective anti-inflammatory status, and its reduction may affect the

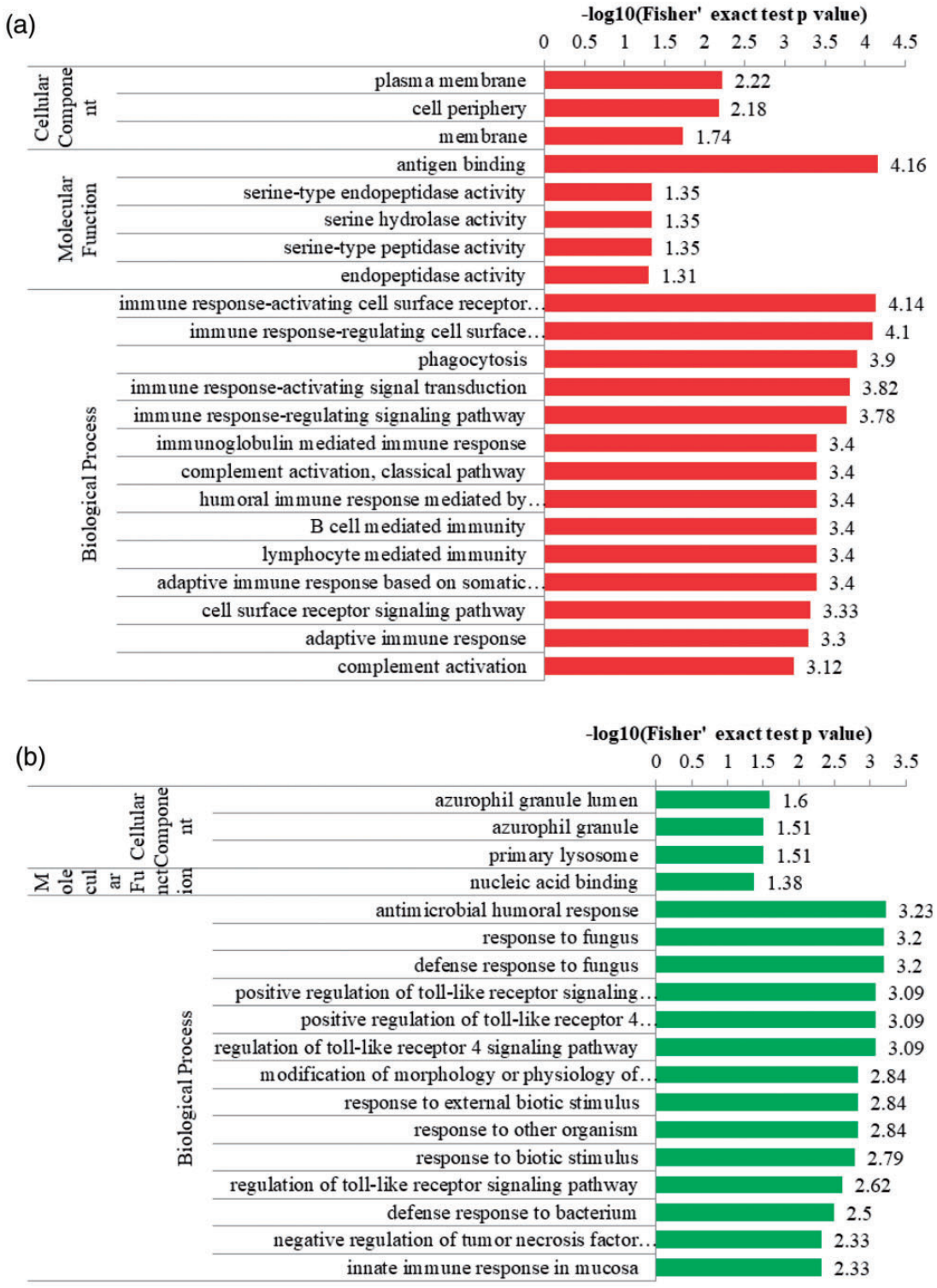

Figure 3. GO enrichment analysis for the dysregulated exo-proteins. (a) GO annotation corresponding to up-regulated exo-protein (>1.2 fold) covering BP, MF, and

normal heart development of the fetus through dysregulated immune regulation and inflammation. Nevertheless, this hypothesis needs further investigation.

While our results suggest that LTF in the serum of the fetus with VSDs is significantly lower than that of a healthy fetus and maternal serum-derived exosomal protein may have potential as a fetus CHD marker in early pregnancy, there are, however, some limitations in this research work. Firstly, the research is limited by its subject size. Protein signatures should be verified in more samples from an independent larger cohort. Multicenter studies with larger samples sizes are necessary to verify the prenatal diagnostic ability of using LTF as a biomarker for diagnosing fetus heart development abnormal diseases before

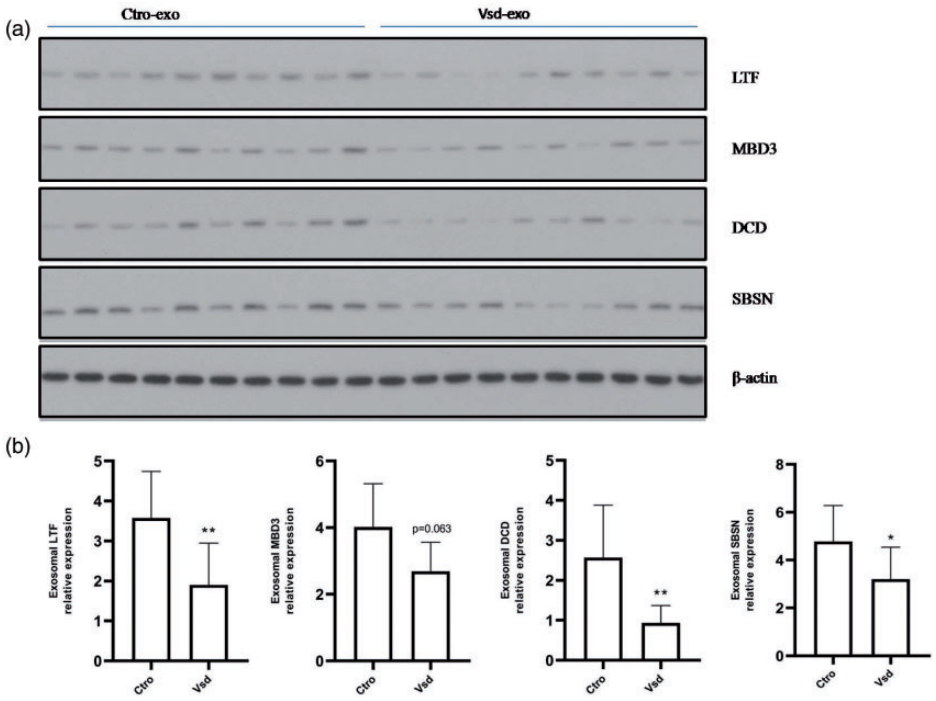

Figure 4. (a) Validation of the differential expression of four select exo-proteins via Western blot analysis: LTF, MBD3, DCD, and SBSN. (b) Data are given as mean ± SEM ($n = 10$).*$p < .05$;**$p < 0.01$ in contrast with the respective controls.

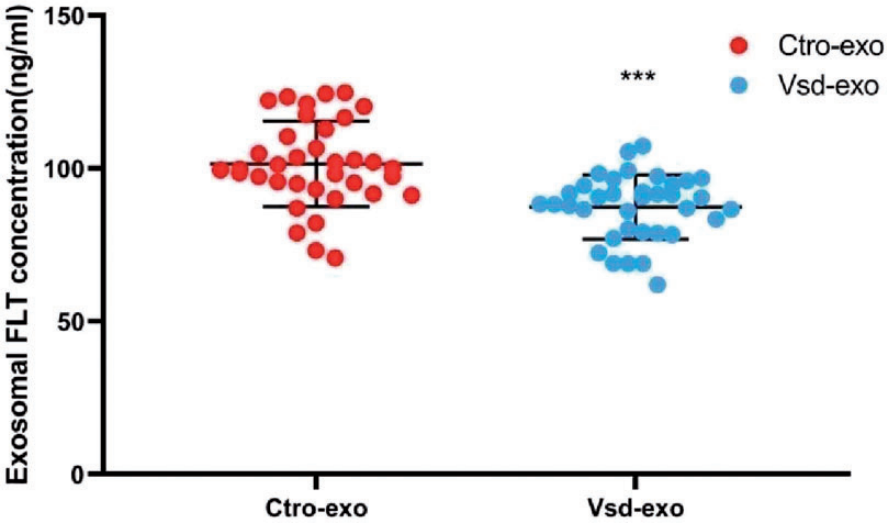

Figure 5. Relative expression levels of exosomal LTF in VSD and healthy controls ($p < 0.001$). (A color version of this figure is available in the online journal.)
VSD: ventricular septal defect.

transition into clinical utility. Secondly, because the standardized approaches of isolation, as well as purification of human fetal-derived exosomes have not been published, only the total levels of serum originated exosomes in pregnant women are detected in our study. Hence, the origin of protein biomarkers is worthy of further research and exploration. However, the association between exosomal protein LTF and fetal VSD has been confirmed by our research. Further studies are needed to prove the potential biological role of exosomal proteins in fetal heart development.

Conclusions

In summary, herein, we discovered, for the first time, the exosomes from VSDs with a low level of LTF may predict the occurrence of fetus VSD in the first trimester of pregnancy. These results are valuable for understanding the

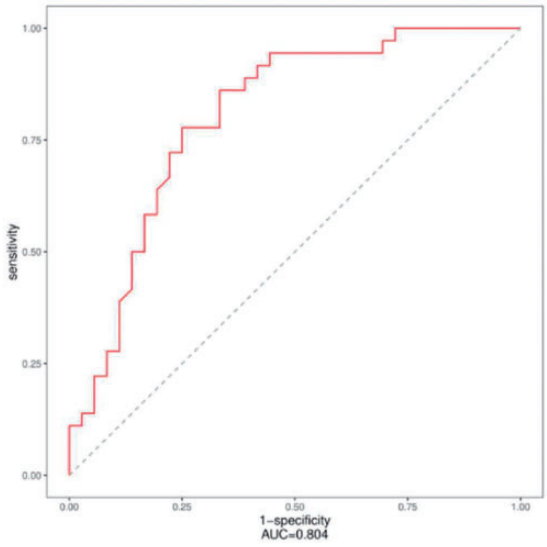

Figure 6. ROC curve exhibiting differences between VSD group and control group (By ROC curve (AUC) analysis, the expression values of the exo-protein LTF resulted in good predictive accuracy). (A color version of this figure is available in the online journal.)
ROC: receiver-operating-characteristic; VSD: ventricular septal defect.

pathogenesis of fetus-VSD. Larger studies are necessary to explore if the identified novel biomarkers can be clinically applied across different CHD phenotypes.

AUTHORS' CONTRIBUTIONS

SL, YJ, and FJ contributed to the conception and design of experiments, authored, as well as reviewed drafts of the paper, PT, XL, and XCh performed the experiments, prepared figures along with the tables, JD and XCh analyzed the data, QZh and MN analyzed the data, prepared figures along with the tables, All authors reviewed previous versions of the manuscript, and read and approved the final version of the article.

ACKNOWLEDGMENTS

The authors thank Dr. Xiaoyang Li (Zhejiang Biosan Biochemical Technologies, Hangzhou 518083, China) for assistance in drawing the figures.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The Jiaxing University Affiliated Women and Children Hospital granted approval of the study. The subjects granted their written informed consent to participate in this study.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and publication of this article: This study was funded by the grants from the National Key R&D Program of China (2018YFC1004900), the Public Welfare Technology Research Program of Zhejiang Province (LGF19H040005 and LGF18H040008), Health Development Planning Commission of Zhejiang Province (2019KY221, 2021KY1120 and 2021KY357), Technology Bureau of Jiaxing, Zhejiang Province (2020AD10030), Research Fund for Academician Lin He New Medicine (19331201 and 19331202).

DATA AVAILABILITY

The original data analyzed in the research work are included in the article. Supplementary Material along with further inquiries can be directed to the corresponding authors. The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium via the PRIDE partner repository with the dataset identifier PXD026616.[22]

ORCID ID

Suping Li https://orcid.org/0000-0002-0463-4380

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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(Received July 18, 2021, Accepted October 26, 2021)