Serum exosomal coronin 1A and dynamin 2 as neural tube defect biomarkers

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Abstract
No highly specific and sensitive biomarkers have been identified for early diagnosis of neural tube defects (NTDs). In this study, we used proteomics to identify novel proteins specific for NTDs. Our findings revealed three proteins showing differential expression during fetal development. In a rat model of NTDs, we used western blotting to quantify proteins in maternal serum exosomes on gestational days E18, E16, E14, and E12, in serum on E18 and E12, in neural tubes on E18 and E12, and in fetal neural exosomes on E18. The expression of coronin 1A and dynamin 2 was exosome-specific and associated with spina bifida aperta embryogenesis. Furthermore, coronin 1A and dynamin 2 were significantly downregulated in maternal serum exosomes (E12–E18), neural tubes, and fetal neural exosomes. Although downregulation was also observed in serum, the difference was not significant. Differentially expressed proteins were further analyzed in the serum exosomes of pregnant women during gestational weeks 12–40 using enzyme-linked immunosorbent assays. The findings revealed that coronin 1A and dynamin 2 showed potential diagnostic efficacy during gestational weeks 12–40, particularly during early gestation (12–18 weeks). Therefore, these two targets are used as candidate NTD screening and diagnostic biomarkers during early gestation.

Key messages
- We used proteomics to identify novel proteins specific for NTDs.
- CORO1A and DNM2 showed exosome-specific expression and were associated with SBA.
- CORO1A and DNM2 were downregulated in maternal serum exosomes and FNEs.
- CORO1A and DNM2 showed good diagnostic efficacy for NTDs during early gestation.
- These two targets may have applications as NTD screening and diagnostic biomarkers.

Keywords Neural tube defects · Spina bifida aperta · Prenatal diagnosis · Proteomics · Exosome

Introduction
Neural tube defects (NTDs) are common congenital malformations that cause severe damage to the fetus as a result of the failure of neural tube closure at 21–28 days after conception [1]. Spina bifida aperta (SBA) is one of the most common types of NTDs and presents with urinary and neurological complications [2]. Moreover, after surgery, long-term follow-up therapy and medical support may be necessary [3].

Ultrasound is one of the most powerful diagnostic tools for NTDs [4]; however, this approach is less effective for identifying NTDs in low-risk pregnancies, particularly...
during the first trimester [5], because NTDs can only be detected after defect formation is complete. The American Congress of Obstetricians and Gynecologists recommends maternal serum alpha-fetoprotein (MSAFP) screening and specialized ultrasound examination to be offered to all pregnant women and those found at high risk for NTDs to identify the defect [6]. Unfortunately, the MSAFP detection rate is only approximately 65–80% when using a cutoff of 2.5 multiples of the median because of various factors [7]. Elevated MSAFP can also be caused by conditions such as fetal abdominal wall defects, congenital kidney disease, fetal death, placenta accreta, preeclampsia, and oligohydramnios [8, 9]. We previously combined the analysis of serum complements (including complement C1q A chain, complement C1s, and complement C3) and MSAFP to overcome the shortcomings of MSAFP analysis and improve the sensitivity and specificity of NTD screening [10]. Indeed, the identification of prenatal molecular biomarkers, particularly specific and sensitive maternal serum biomarkers, may facilitate the early screening, diagnosis, and treatment of NTDs during early gestation.

Extracellular vesicles (EVs) can pack and preserve proteins, RNA, and DNA, enrich and transfer informative factors with more specificity, and are not easily degraded in bodily fluids [11]. In particular, exosomes permit the specific transport of cargo to target cells during pregnancy [12] and have been shown to be more stable than the other types of EVs [13]. Exosomes can be detected at high levels during gestational week 6 [14], and the contents of these EVs are altered under pathological conditions [15]. Exosomes have been found in the placenta [16] and amniotic fluid [17] and can transfer to the maternal side [18]. Indeed, approximately 35% of total maternal exosomes are fetal [19]. Furthermore, fetal neural exosomes (FNEs) change when exposed to ethyl alcohol [20, 21]. Therefore, exosomes may carry specific molecular biomarkers from the fetus to the mother and may have potential applications in prenatal screening and diagnosis.

Proteomics is a powerful tool that can identify specific biomarkers of NTDs and their underlying molecular mechanisms in relevant animal models and clinical samples [22]. We previously found 14–3-3ζ present distinctly in fetal neural tubes [23], apolipoprotein A4 and alpha-fetoprotein (AFP) fragment changes in amniotic fluid [24], and proprotein convertase subtilisin/kexin type 9 changes in serum [25]. However, no differentially expressed exosomal proteins have been identified as diagnostic biomarkers of NTDs.

Accordingly, in this study, we used proteomics and bioinformatics to screen for enriched proteins in maternal serum exosomes as potential SBA biomarkers.

Materials and methods

Clinical sample collection

Serum samples from pregnant women were collected from the biological specimen bank of the Shengjing Birth Cohort in Key Laboratory of Health Ministry for Congenital Malformation, Shenyang, China. Nineteen pregnant women were diagnosed as carrying fetuses with NTDs using prenatal ultrasound, and the diagnosis was confirmed by autopsy after induced labor or by physical examination after delivery. Serum samples from gestational age- and maternal age-matched controls were obtained from pregnant women (n = 19) carrying normal fetuses without any abnormalities. Every pregnant woman consumed folic acid regularly under guidance during pregnancy. The clinical characteristics of the patients are summarized in Online Resource 1.

Animal sample collection

SBA was induced in Wistar rats with all-trans-retinoic acid (atRA; Sigma, St. Louis, MO, USA; 4% [w/v] in olive oil; 140 mg/kg body weight) at E10 (vaginal smear containing sperm designated E0) by gavage, and normal controls were treated with the same volume of oil, as described previously [24, 26]. Briefly, animals were anesthetized with isoflurane, and blood was collected from the apex cordis of living rats; euthanasia was then carried out by CO₂ asphyxiation. All experimental protocols involving animals were approved by the Medical Ethics Committee of the Shengjing Hospital of China Medical University (2016PS106K).

Fetal deformities were examined using stereomicroscopy (M165 FC; Leica, Mannheim, Germany; Online Resource 4. Fig. 1). Blood samples were collected into vacuum tubes (Vacutainer SST; Becton, Dickinson and Company, Franklin Lake, NJ, USA) and centrifuged (Sorvall ST8R Centrifuge; Thermo Fisher Scientific, Walther, MA, USA) at 2000 × g and 4 °C for 20 min for serum sampling. Serum samples were collected from normal pregnant rats with normal embryos at E12 (n = 12), E14 (n = 6), E16 (n = 6), and E18 (n = 25) and from pregnant rats with SBA embryos at E12 (n = 12), E14 (n = 6), E16 (n = 6), and E18 (n = 25). We also collected 12 treated E18 samples from normal (n = 3) rats treated with oil, those without SBA (n = 3) from rats treated with 140 mg/kg atRA, those with SBA (n = 3) from rats treated with 140 mg/kg atRA (SBA group 1), and those with SBA (n = 3) from rats treated with 110 mg/kg atRA (SBA group 2; Online Resource 2). The fetal neural tube tissue (E12) or spinal cord tissue
(E18) (from the inferior margin of the forelimb bud to the tail bud) was isolated in cold phosphate-buffered saline (PBS). Samples were stored at $-80^\circ$C, except tissues used for immunohistochemistry, which were preserved in 4% paraformaldehyde and then embedded in paraffin.

**Total serum exosome extraction and characterization**

A 200-μL aliquot of each serum sample was adjusted to 3 mL with phosphate-buffered saline (PBS) and utilized for extraction. The diluted serum was filtered through a 0.22-μm filter (MILLEX GP, Millipore Express PES Membrane; Millipore, Billerica, MA, USA) [27]. The filtered sample was centrifuged at 10,000×g and 4°C for 1 h. Subsequently, the supernatant was transferred to a fresh tube and centrifuged at 100,000×g and 4°C for 4 h. The pellets were washed with PBS, ultracentrifuged at 100,000×g and 4°C for 1 h again to purify the exosomes [28]. The pellet was resuspended in 100 µL PBS and preserved at $-80^\circ$C as previously described [29, 30]. Characterization of the extracted exosomes was confirmed by transmission electron microscopy (HT7800; Hitachi Koki, Tokyo, Japan), dynamic light scattering (Nano ZS90; Malvern Instruments, UK), and exosomal biomarkers (Alix, CD63, and CD9; Online Resource 4, Fig. 2).

**FNE isolation**

Exosomes from fetal neural sources were isolated as previously described [21, 22]. Briefly, 400 µL sample was incubated for 90 min at 20°C with 50 µL of 3% bovine serum albumin (BSA; Solarbio, Beijing, China) containing 2 µg polyclonal goat IgG anti-rat Contactin-2/TAG1 antibody (AF4439; R&D Systems, Minneapolis, MIN, USA) that had been biotinylated (EZ-Link sulfo-NHS-biotin System; Thermo Fisher Scientific). Then, 10 µL Streptavidin-Plus UltraLink resin (Pierce; Thermo Fisher Scientific) in 40 µL of 3% BSA was added. After centrifugation, the supernatant was transferred to an Eppendorf tube and stored at $-80^\circ$C.

**LC–MS/MS for biomarker screening**

Three pairs of E18 samples were screened for potential biomarkers using label-free LC–MS/MS [31, 32]. Samples were first subjected to immunoaffinity depletion of high-abundance serum proteins. Then, 200 µg protein for each sample was...
processed by filter-aided sample preparation digestion. The peptide content was estimated by determining the ultraviolet spectral density at 280 nm, calculated based on the frequency of tryptophan and tyrosine in vertebrate proteins [32]. Each fraction was then injected for nanoLC-MS/MS analysis. The peptide mixture was separated with a linear gradient controlled by IntelliFlow technology. LC–MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an Easy nLC (Proxeon Biosystems). The MS data were analyzed using MaxQuant software version 1.5.3.17 (Max Planck Institute of Biochemistry, Martinsried, Germany) [33].

Fig. 2 Analyses of ACTR2, CORO1A, and DNM2 expressions in serum exosomes and serum without isolation of exosomes of pregnant rats. a Western blot analysis of protein expression in serum exosomes at E18 (complete bands are shown in Online Resource 4. Fig. 3); bar chart of relative protein expression at E18 in the normal (n = 19) and SBA (n = 19) groups. b Western blot analysis of protein expression in serum exosomes at E16 (complete bands are shown in Online Resource 4. Fig. 4); bar chart of relative protein expression at E16 in the normal (n = 6) and SBA (n = 6) groups. c Western blot analysis of protein expression in serum exosomes at E14 (complete bands are shown in Online Resource 4. Fig. 5); bar chart of relative protein expression at E14 in the normal (n = 6) and SBA (n = 6) groups. d Western blot analysis of protein expression in serum exosomes at E12 (complete bands are shown in Online Resource 4. Fig. 6); bar chart of relative protein expression at E12 in the normal (n = 12) and SBA (n = 12) groups. e Western blot analysis of protein expression in serum without isolation of exosomes at E18 (complete bands are shown in Online Resource 4. Fig. 7); bar chart of relative protein expression in serum without isolation of exosomes at E18 in the normal (n = 6) and SBA (n = 6) groups. f Western blot analysis of protein expression in serum without isolation of exosomes at E12 (complete bands are shown in Online Resource 4. Fig. 8); bar chart of relative protein expression in serum without isolation of exosomes at E12 in the normal (n = 6) and SBA (n = 6) groups.

Bioinformatics analysis

Bioinformatics analysis was carried out using WebGestalt (http://www.webgestalt.org/) with over-representation analysis (ORA) in Rattus norvegicus for enrichment with the gene ontology (GO) biological process functional database. The significance level in the advanced parameters was adjusted to a false-discovery rate of less than 0.05. The proteins showing enrichment were further analyzed using the String database (https://string-db.org/) to define protein interaction networks.
Western blotting

Exosome-derived from serum (E12, E14, E16, E18), serum (E12, E18), neural tube tissue (E12), and spinal cord tissue (E18) were treated with 20 µL radioimmunoprecipitation assay buffer (Solarbio) per 100 µL sample and subjected to ultrasound pyrolysis. The supernatants were collected as the protein-containing fraction. Serum (E12, E18) was diluted by

Table 1: List of 33 differentially expressed proteins

| Protein IDs  | Protein names                              | Gene names | Ratio |
|--------------|--------------------------------------------|------------|-------|
| Upregulated  | Complement C1s subcomponent C1s            |            | 2.99  |
| F1LZ11       | Uncharacterized protein                     | N/A        | 0.65  |
| Q62636       | Ras-related protein Rap-1b                 | Rap1b      | 0.65  |
| P01015       | Angiotensinogen                            | Agt        | 0.63  |
| D3ZPL2       | Uncharacterized protein                     | N/A        | 0.59  |
| P09606       | Glutamine synthetase                       | Glul       | 0.55  |
| F1M5X4       | Ig-like domain-containing protein           | N/A        | 0.55  |
| P68136       | Actin, alpha skeletal muscle                | Acta1      | 0.54  |
| Q8K3U6       | Coagulation factor VII                     | F7         | 0.53  |
| A0A0G2J6V65  | 14–3-3 protein zeta/delta                  | Ywhaz      | 0.52  |
| B0BNJ1       | LOC683667 protein                          | Sri        | 0.50  |
| A0A0G2K9Z5   | Uncharacterized protein                     | N/A        | 0.48  |
| G3V7W1       | Programmed cell death protein 6             | Pdcd6      | 0.45  |
| P01883       | Ig delta chain C region (fragment)          | N/A        | 0.09  |

Table 2: List of gene ontology analyses of the biological processes associated with the 29 differentially expressed proteins

| Gene set        | Description                                         | Size | Expect | Ratio   | P value   | FDR    |
|-----------------|-----------------------------------------------------|------|--------|---------|-----------|--------|
| GO:0008064      | Regulation of actin polymerization or depolymerization | 122  | 0.2374 | 21.058  | 3.30E–06  | 0.005217|
| GO:0022603      | Regulation of anatomical structure morphogenesis     | 682  | 1.3273 | 6.7805  | 3.19E–06  | 0.005217|
| GO:1901700      | Response to oxygen-containing compound               | 1542 | 3.0011 | 4.3317  | 1.50E–06  | 0.005217|
| GO:0022607      | Cellular component assembly                          | 1997 | 3.8867 | 3.6021  | 4.34E–06  | 0.00563 |
Analyses of ACTR2, CORO1A, and DNM2 expressions in spinal cords, neural tubes, and FNEs. a Western blot analysis of protein expression in spinal cords at E18 (complete bands are shown in Online Resource 4; Fig. 9); bar chart of relative protein expression at E18 in the normal ($n=6$) and SBA ($n=6$) groups. b Western blot analysis of protein in neural tubes at E12 (complete bands are shown in Online Resource 4; Fig. 10); bar chart of relative protein expression at E12 in the normal ($n=6$) and SBA ($n=6$) groups. c Figures above: expression of ACTR2 in the spinal cord of normal embryos, localized to the neuroepithelium (NE), neural cells (NCs), and neural fibers (NFs) (black arrow), expression of CORO1A in the spinal cord of normal embryos, localized to NFs and NCs (black arrow), expression of DNM2 in the spinal cord of normal embryos, localized to the NE and NCs (black arrow); figures below: expression of ACTR2, CORO1A, and DNM2 in the spinal cord of SBA embryos. d Western blot analysis of ACTR2, CORO1A, and DNM2 expressions in FNEs at E18 in the normal and SBA groups; bar chart of relative protein expression in FNEs at E18 in the normal ($n=3$) and SBA ($n=3$) groups, as determined by western blotting.

PBS before western blotting. Samples were quantified using a BCA Protein Assay Kit (Solarbio), and protein concentrations were adjusted using PBS. The diluted samples were mixed with loading buffer, denatured, separated on Bio-Rad gels, and transferred to 0.45-μm polyvinylidene fluoride membranes. The primary antibodies used were as follows: anti-Alix (3A9 mouse mAb; Cell Signaling Technology, Danvers, MA, USA; exosome internal reference), anti-CD9 (D3H4P rabbit mAb; Cell Signaling Technology), anti-CD63 (TS63 mouse mAb; Abcam, Cambridge, UK), anti-β-actin (66009–1-lg rabbit mAb; Proteintech, Wuhan, China; neural tube internal reference), anti-actin-related protein 2 (ACTR2; 10922–1-AP rabbit pAb; Proteintech), anti-coronin 1A (CORO1A; EPR19467-36 rabbit mAb; Abcam), and anti-dynamin 2 (DNM2; EPR9053 rabbit mAb; Abcam). The secondary antibodies used were goat anti-mouse (G-21040; Invitrogen, Carlsbad, CA, USA) or goat anti-rabbit (G-21234; Invitrogen). Fast-staining Coomassie Brilliant Blue (Solarbio) served as an internal reference for serum. Bands were visualized using chemiluminescent horseradish peroxidase-based substrate (Immobilon Western; Millipore) and captured (cSeries 300; Azure Biosystems, Dublin, CA, USA). Representative samples are shown, and raw data for the bands are shown in the Online Resource 4.

Immunohistochemistry

An UltraSensitive SP Kit (MXB Biotechnologies, Fuzhou, China) was used for immunohistochemistry, according to the manufacturer’s protocol. The primary antibodies were the same as used for western blotting. Sections were washed in PBS between each process and finally stained with diamobenzidine and hematoxylin, dehydrated, and sealed with resin. Images were captured using a microscope (ECLIPSE 80i; Nikon, Tokyo, Japan).

ELISA

Serum (50 μL) was diluted with PBS (50 μL), and samples were subjected to ELISA using a human DNM2 ELISA kit (abx250599; 96-well; Abbexa) and human CORO1A ELISA kit ab214032; 96-well; Abcam), according to the manufacturer’s protocol. Absorbance was measured at 450 nm using a microplate reader (M200 PRO; Tecan, Switzerland). The relative optical density at 450 nm (OD$_{450}$; OD$_{450}$ of each well–OD$_{450}$ of the blank well) was calculated.

Statistical analysis

Data are expressed as means ± standard deviations. Western blotting bands were quantified using ImageJ (1.37c) as the ratio of the gray value of the biomarker protein/the gray value of the internal reference protein. Quantitative variables were analyzed using unpaired t-tests, paired t-tests, and one-way analysis of variance (ANOVA). Differences with P values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 8.0 software. The diagnostic capacity of biomarkers was analyzed using receiver operating characteristic (ROC) curves, and the area under curve (AUC), specificity, and sensitivity were determined using MedCalc 19.3.1 in Statistics-ROC.

Results

Identification of serum exosome protein biomarkers of SBA using LC–MS/MS

Proteomics analyses revealed the presence of 397 proteins in serum exosomes (Online Resource 3), of which 33 were differentially expressed between the SBA and normal groups. There were 7 proteins specifically expressed in the SBA group and 12 proteins specifically expressed in the normal group excluding potential contaminants. Another 14 proteins were expressed in both the SBA and normal groups with a significant difference (one upregulated and 13 downregulated; Table 1). GO analysis was conducted to identify the potential functions of these proteins (excluding four uncharacterized proteins) in three categories (Fig. 1a), and ORA was used to obtain GO enrichments of biological processes with four enrichment processes (Table 2). ACTR2, CORO1A, DNM2, angiotensinogen precursor, glutamate-ammonia ligase (GLUL), LIM zinc finger domain containing 1, programmed cell death 6, and pyruvate kinase muscle isozyme were involved in more than two biological processes.

Among these eight proteins, ACTR2 [34, 35], CORO1A [36], GLUL [37], and DNM2 [38] function during neural tube development but have not been shown to be connected with NTDs. Analysis of the four proteins in the String
ANALYSIS OF THE DIFFERENTIAL EXPRESSION OF ACTR2, CORO1A, AND DNM2 IN SERUM WITHOUT ISOLATION OF EXOSOMES

To confirm the specific differential expression of these proteins in serum exosomes, we quantified their expression in whole serum without exosome isolation using western blotting. We found no obvious changes between the normal and SBA groups at E18 (Fig. 2e) and E12 (Fig. 2f), indicating their serum exosome-specific downregulation. Coomassie Brilliant Blue staining of total proteins served as an internal reference for serum (complete gel staining is shown in Online Resource 4. Figs. 7, 8). Unpaired t-tests supported that there were no significant differences at E18 (ACTR2, P = 0.5082; CORO1A, P = 0.7230; DNM2, P = 0.1316) (Fig. 2e) or E12 (ACTR2, P = 0.6568; CORO1A, P = 0.3789; DNM2, P = 0.8318) (Fig. 2f).

ANALYSIS OF THE DIFFERENTIAL EXPRESSION OF ACTR2, CORO1A, AND DNM2 IN FETAL NEURAL TUBES AND FNEs

To verify whether the downregulation of these proteins in serum exosomes was related to NTDs, we quantified their expression in SBA spinal cords at E18 (Fig. 3a) and neural tubes at E12 (Fig. 3b) using western blotting. The expression patterns were the same as in serum exosomes, showing significantly decreased expression in SBA. Unpaired t-tests supported these findings at E18 (ACTR2, P = 0.0012; CORO1A, P = 0.0264; DNM2, P = 0.0151) (Fig. 3a) and E12 (ACTR2, P = 0.0301; CORO1A, P = 0.0247; DNM2, P = 0.0012) (Fig. 3b).

Immunohistochemistry was performed in E18 embryos to evaluate the localization and expression levels of these proteins in neural tubes. ACTR2 was expressed in all cells within the field of vision, with upregulation in the neuroepithelium, neural cells, and neural fibers in normal control and downregulation in SBA. CORO1A was upregulated in nerve fibers in normal control but downregulated in SBA. DNM2 was upregulated in the neuroepithelium and neural cells in normal control but hardly detected in SBA (Fig. 3c).
To further verify whether ACTR2, CORO1A, and DNM2 downregulated in total serum exosomes originated from the fetal neural source, we tested the proteins in FNEs isolated from total serum exosomes of maternal rats. Changes in CORO1A and DNM2 expressions in FNEs followed the same pattern as in total serum exosomes, with downregulation in SBA (CORO1A, \( P = 0.0487 \); DNM2, \( P = 0.0316 \)); ACTR2 expression did not differ between normal samples and SBA FNEs samples (ACTR2, \( P = 0.4987 \)) (Fig. 3d). Thus, CORO1A and DNM2 expressions were specific to neural cells and tissues.

**Analysis of the differential expression of CORO1A and DNM2 in serum exosomes from pregnant women**

Next, we performed ELISA of DNM2 and CORO1A in maternal serum exosomes to validate their expression. Samples from pregnant women were paired according to gestational week, and paired \( t \)-tests were used for statistical analysis. Compared with normal controls, all 19 pregnant women who were diagnosed as carrying fetuses with NTDs showed a significant decrease in DNM2 expression (\( P = 0.0011 \)) (Fig. 4a), and the ROC curve showed high accuracy (specificity, 78.95%; sensitivity, 73.68%; AUC, 0.806) (Fig. 4b). Analysis during different pregnancy periods showed that DNM2 was significantly downregulated at gestational weeks 12–18 (\( P = 0.0034 \)) (Fig. 4c), and this marker showed extremely high accuracy (specificity, 100%; sensitivity, 100%; AUC, 1.000) (Fig. 4d). Downregulation was not significant at gestational weeks 19–40 (\( P = 0.0557 \)) (Fig. 4e). Subgroup analysis showed significant differences in DNM2 expression in the SBA group (\( P = 0.0035 \)) (Fig. 4f) and the anencephalus and exencephalus group (\( P = 0.0058 \)) (Fig. 4g).

CORO1A expression was also significantly downregulated in the 19 pregnant women compared with that in normal controls (\( P = 0.0022 \)) (Fig. 4h), and its ROC curve showed high accuracy (specificity, 89.47%; sensitivity, 68.42%; AUC, 0.817) (Fig. 4i). Furthermore, significant downregulation was observed at gestational weeks 12–18 (\( P = 0.0108 \)) (Fig. 4j), and this marker showed high accuracy (specificity, 85.71%; sensitivity, 85.71%; AUC, 0.857) (Fig. 4k). No significant downregulation was observed at gestational weeks 19–40 (\( P = 0.0599 \)) (Fig. 4l). Subgroup analysis showed that CORO1A was differentially expressed in the SBA group (\( P = 0.0440 \)) (Fig. 4m) and the anencephalus and exencephalus group (\( P = 0.0186 \)) (Fig. 4n).

We further analyzed the combined performance of DNM2 and CORO1A in diagnostic efficacy of NTDs. The ROC curve showed better performance in all 19 pair samples and exhibited higher accuracy (specificity, 78.95%; sensitivity, 94.74%; AUC, 0.889) (Fig. 4o). At gestational weeks 12–18, ROC curves showed better performance and high accuracy (specificity, 100%; sensitivity, 100%; AUC, 1.000) (Fig. 4p). At gestational weeks 19–40 (\( n = 12 \)), ROC curves showed good performance (specificity, 66.67%; sensitivity, 100%; AUC, 0.854) (Fig. 4q). ROC curves also showed good performance in 13 paired SBA samples, with high accuracy and specificity (specificity, 92.31%; sensitivity, 76.92%; AUC, 0.888) (Fig. 4r).

**Discussion**

In this study, we showed that ACTR2, CORO1A, and DNM2 were downregulated in serum exosomes in a SBA rat model using LC–MS/MS (label-free) in conjunction with bioinformatics analyses. CORO1A and DNM2 were also differentially expressed in FNEs, and these findings were validated in pregnant women, indicating that these two proteins may be candidate specific diagnostic biomarkers of NTDs during early gestation.

All three of these proteins have crucial roles in fetal development. CORO1A is involved in axon guidance and branching to final targets by mediating actin assembly and reorganization with cofilin and the ARP2/3 complex (ARPC3) \[36\]. CORO1A also stabilizes \[39\] and binds to \[40\] F-actin. The large GTPase DNM2 controls the spreading and motility of the growth cone \[38\] and can regulate hormone secretion and vesicle release from neuroendocrine cells \[41\]. F-actin requires DNM2 to polymerize and assemble with ARPC3 \[42\]. Moreover, reorganization of F-actin requires the DNM2/cortactin/ARPC3 complex \[43\]. ACTR2, a component of ARPC3, is essential during different phases of neural development, including neurogenesis, neuritogenesis, and neural migration \[44\]. ARPC3 is also critical for the regulation and dynamics of F-actin \[35, 45, 46\], which is involved in axon and dendrite growth \[47, 48\] as well as neural tube closure \[49, 50\]. Therefore, F-actin may be an effective indicator of neural development associated with these molecules.

There were some limitations to our study. First, the routine administration of folic acid during gestation has substantially reduced the occurrence of NTDs; therefore, the sample size of pregnant women carrying fetuses with NTDs was quite small. More data from clinical samples are required to confirm our findings and perform combined analysis of the potential biomarkers. Additionally, the earliest samples of NTDs were from gestational week 12, and further studies should verify whether CORO1A and DNM2 can be used to screen and diagnose NTDs before gestational week 12. Multicenter studies are also needed to validate the prenatal diagnostic potential of these biomarkers, and additional work is necessary to identify the detailed mechanisms through which CORO1A and DNM2 are related to embryogenesis in SBA.

CORO1A and DNM2 expressions in exosomes extracted from maternal serum during pregnancy may have...
applications in the early clinical screening and diagnosis of NTDs with high specificity. Our study established these novel and candidate molecular biomarkers and suggested their involvement in the occurrence of NTDs.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s00109-022-02236-w.

Author contribution Yanfu Wang, Ling Ma, and Zhengwei Yuan designed this study. Yanfu Wang performed the experiments with the help of Ling Ma. Yanfu Wang acquired the data. Yanfu Wang analyzed the data with the help of Shanshan Jia, Dan Liu, Wei Ma, Xiaowei Wei, and Zhengwei Yuan. Hui Gu, Wenting Luo, Yuzuo Bai, and Weilin Wang provided critical revisions. Yanfu Wang drafted the manuscript. All authors read and approved the final manuscript.

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Data availability The raw dataset for LC–MS/MS (label-free) is accessible on the Proteomics IDEntifications Database (https://www.ebi.ac.uk/pride/). URL: https://www.ebi.ac.uk/pride/archive/projects/PXD023775/private, project ID: PXD023775).

Declarations

Ethics approval Every pregnant woman consumed folic acid regularly under guidance during pregnancy. All pregnant women were informed of the study procedures and risks, and all participants consented to participate in this study. Written informed consent was obtained from all pregnant women at the time of sampling, and the study was performed in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Shengjing Hospital Affiliated to China Medical University (Approval no. 2017PS264K). All experimental protocols involving animals were approved by the Medical Ethics Committee of the Shengjing Hospital of China Medical University (Approval no. 2016PS106K).

Competing interests The authors declare no competing interests.

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