

VDAC1 and RhoA in Plasma Exosomes Influence the Severity of Adolescent Idiopathic Scoliosis

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Abstract

Background: Adolescent idiopathic scoliosis (AIS) is the most common spine deformity, but biomarkers for its condition are lacking. Rhodopsin A (RhoA) and voltage-dependent anion-selective channel 1 (VDAC1) in plasma exosomes were defined as differentially expressed proteins between AIS patients and healthy controls. The purpose of this study was to assess exosomes as biomarkers for the occurrence and progression of AIS.

Methods: We recruited 10 AIS patients and 8 healthy controls to detect expressed proteins from plasma by liquid chromatography coupled to tandem mass spectrometry. Plasma samples were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Pathway analysis identified that the VDAC1 and RhoA proteins were alterations expressed in the AIS patients, with the most different alteration was found in extracellular exosomes. Ultracentrifugation was carried out to isolate exosomes from plasma. Verification of the most differentially expressed protein was accessed by Western blot analysis and bioinformatics analysis was performed to predict the pathway of it.

Results: 42 of significantly differentially expressed proteins were found in all subjects, and 17 proteins had significant difference. The differentially expressed proteins were enriched in plasma exosomes, and some proteins, such as FN1, were upregulated and others, such as VDAC1, RhoA and AHNAK, were downregulated in the AIS patients. Furthermore, ultracentrifugation was carried out to isolate exosomes from plasma, and RhoA and VDAC1 proteins in plasma exosomes were verified to downregulate by western blot. KEGG signaling pathways were used to predict potential pathways involved in the RhoA and VDAC1 proteins in the AIS patients. We found that the RhoA protein influences AIS probably through the chemokine signaling pathway, platelet activation and cAMP signaling pathway, and the VDAC1 protein is a key factor that participates in the necroptosis pathway, acting on the development of AIS.

Conclusions: Consequently, this study mapped a profile of plasma protein, found the differentially expressed protein in AIS, which indicating that plasma exosomes, as a novel biomarker with high specificity, could be associated with the severity of AIS.

Background

Adolescent idiopathic scoliosis (AIS) is defined as a lateral curvature of the spine with a Cobb angle of at least 10° ^[1] and affects approximately 0.47–11.1% of the general population^[2]. The reported prevalence was 1.8%^[3]. AIS is a highly heterogeneous condition, with some patients showing rapid progression^[4]. This disease increases the risk of physical and mental symptoms, such as trunk deformity and pulmonary impairment^[5, 6]. Several previous studies failed to identify biomarkers of AIS^[7, 8]. Therefore, screening biomarkers that can be used in the early identification and diagnosis of AIS is important for monitoring curvature progression before skeletal maturation.

Exosomes are 20–100 nm in size and are enveloped by a phospholipid membrane. These particles are produced by normal as well as malignant cells and are present in all human body fluids^[9]. Exosomes are considered a biomarker for many diseases^[10–14]. Similar to cells, exosomes are composed of lipid bilayers and contain many molecular components of cells, such as proteins, miRNAs, and DNA^[15]. We used proteins to confirm the role of exosomes because they usually display a high purity^[16] and stable expression.

In this study, in order to investigate novel biomarkers of AIS, we used mass spectrometry (MS) to screen the differentially expressed plasma proteins between AIS patients and healthy controls, and the potential biomarkers of exosomal proteins in AIS patients were confirmed.

Methods

Patients and clinical samples

All the participants provided written informed consent. A total of 10 AIS patients and 8 matched healthy individuals with similar baselines (Table 1) were enrolled from the Xinhua Hospital affiliated with the Shanghai Jiaotong University School of Medicine from August 2017 to August 2018. According to The Scoliosis Research Society (SRS), all enrolled AIS patients were confirmed when the Cobb angle was 10° or higher and axial rotation was observed by radiography and a physical examination. The study processes are shown in Fig. 1.

Table 1
Clinical characteristic of the recruited subjects in clinical study.

Characteristics	Groups	
	AIS (N = 10)	Control(N = 8)
Age(year)	13.60 ± 1.17	14.88 ± 0.83
Female(case)	9(1)	7(1)
BMI(kg/m ²)	18.64 ± 1.66	19.31 ± 1.99
Menarche age (year)	11.89 ± 1.36	12.2 ± 0.76
Cobb angle		
10°-50°	10	-
≥ 50°	0	-
Risser		
0-3	7	-
4-5	3	-
Note: BMI, body mass index.		

Ten milliliters of venous blood were collected in EDTA anticoagulant tubes. Plasma was obtained after a 15-min centrifugation at 300 g to remove the cells and a second 5-min centrifugation at 1500 g to remove the remaining platelets and apoptotic bodies. The purified plasma samples were stored at -80 °C until further use.

Isolation of the exosomes from the blood plasma with ultracentrifugation

After centrifugation at 2,000 g and 12,000 g to remove the cells and cellular debris, the exosomal supernatant was centrifuged at 110,000 g for 2 hours to collect the exosomal fraction. Then, the suspension was filtered through a 0.22 µm filter, centrifuged at 110,000 g for 70 min two times, and resuspended in PBS. All centrifugation steps were performed at 4 °C.

Flow cytometry and analysis of particle size

To confirm the contents of the purified exosomes from the plasma samples, we used an Accuri C6 flow cytometer (BD Instruments) to detect CD63 and CD81 by flow cytometric analysis. The size distribution of both kinds of exosomes was evaluated with a Zetasizer Nano series-Nano-ZS (Malvern Instruments).

Electron microscopy

Fifty microliters of exosomal proteins was adsorbed by copper grids and fixed with 1% glutaraldehyde. After staining with 3% phosphotungstic acid solution, the grids and the samples were dried, and a JEM1230 system was used to image the exosome samples.

LC-MS/MS

Sample dilution buffer (100 mM, 10 mM HEPES, pH = 7.4) was added to the blood sample to remove the lipids, and then, cold ethanol solution was used to remove the peak components. Next, we enzymatically hydrolyzed the protein sample by TEAB solution containing trypsin. Then, label reagent was added to each tube, and the sample corresponding to each TMT color was recorded and mixed. The TMT-labeled peptide mixture was separated on an Agilent 1260 HPLC system at a rate of 0.2 ml/min using a Water XBridge BEH130 C18 3.5 μ m 2.1A ~ 150 mm column, and the CBS-B programmed multifunctional automatic fraction collector (Huxi Instruments, Shanghai, China) for HPLC was used to collect the eluted peptides. A total of 28 fragments of each peptide mixture were collected, ligated to level 14, dried and subjected to nano LC-MS/MS analysis. An Easy nano-LC (Thermo Fisher Scientific) system was inverted at a flow rate of 300 nl/min using a self-packing column (75 μ m \AA ~150 mm; 3 μ m ReproSil-Pur C18 beads, 120 \AA , Dr. Maisch GmbH, Ammerbuch, Germany) for high-performance liquid chromatography (RP-HPLC) separation. The MS analysis detected the parameters of the 12 strongest ions and scanned the results by an Orbitrap analyzer.

Western blotting

Exosomal proteins (15–20 μ l, 30 μ g) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (GE Healthcare). After the membranes were blocked with 5% nonfat milk in PBST buffer (PBS buffer containing 0.1% Tween-20), they incubated in anti-calnexin (1:1000, Abcam), anti-CD63 (1:4000, Abcam) and anti-TSG101 (1:4000, Abcam) antibodies for verified exosome-specific markers. Anti-voltage-dependent anion-selective channel 1 (VDAC1) (1:1000, Abcam), anti-rhodopsin (RhoA) (1:1000, Abcam), anti-AHNAK (1:2000, Abcam) and anti-fibronectin (FN1) (1:12000, Abcam) were incubated overnight at 4°C. Then, the membranes were washed and exposed to the corresponding secondary antibody–rabbit IgG HRP-linked antibody (1:2000, Cell Signaling) and mouse IgG HRP-linked antibody (1:2000, Cell Signaling)—for 1 hour at room temperature and washed 3 times for 15 min in wash buffer. Finally, we detected the protein bands with enhanced chemiluminescence reagent (Millipore) and imaged them with an ImageQuant LAS 4000 mini densitometer (GE Healthcare Life Sciences).

Bioinformatics and statistical analyses

Functional gene ontology enrichment analysis of biological processes, molecular functions, and cellular components was performed using DAVID Bioinformatics Resources version 6.7. The protein-protein interaction (PPI) network of differentially expressed proteins was analyzed by STRING. The PPI network was processed by Cytoscape software. The pathway network was further analyzed by Ingenuity Pathway Analysis (IPA).

All statistical analyses were performed using SPSS 20.0 statistical software. Data are expressed as the mean \pm standard deviation. The Pearson correlation test was used for the correlation between the Cobb angle and the differentially expressed proteins. Any difference with a P value less than 0.05 after correction was considered significant.

Results

Exosomes proteins were differentially expressed in the AIS patients and the controls

After the exosomes were isolated from the plasma of the AIS patients and the controls, we performed MS to screen the differentially abundant proteins in the exosomes of the plasma samples from the AIS patients versus the healthy controls (\log_2 ratio > 1.5 or < -1.5). A total of 652 proteins were identified, and 626 proteins had quantitative information. Forty-two significantly differentially expressed proteins were found in the AIS group and the control group, and 17 proteins had values greater than 1.5 (Fig. 2A, B) (Table 2).

Table 2
The characteristics of differential proteins (larger than 1.5).

Proteins	Regulation	Position	Functions
FN1	Up regulation	Extracellular matrix	Participate in cell adhesion, cell motility, opsonization, wound healing, maintenance of cell shape, osteoblast mineralization
UCP3	Up regulation	Mitochondrion inner membrane	As a mitochondrial transporter proteins participate in thermogenesis and energy balance
IGHV1OR15-9	Up regulation	-	Immunoglobulin receptor binding
IGHV1-69	Up regulation	Cell membrane	Antigen recognition, immunoglobulins
ISY1	Up regulation	Nucleus	Component of the spliceosome
IGKV1-33	Up regulation	Cell membrane	Antigen recognition, immunoglobulins
CELSR2	Up regulation	Cell membrane, Cytosol	Receptor in cell/cell signaling during nervous system formation
IGLV3-10	Up regulation	Cell membrane	Antigen recognition, immunoglobulins
IGHV1-3	Up regulation	Cell membrane	Antigen recognition, immunoglobulins
ENO1	Down regulation	Cytoplasm, Cell membrane	Multifunctional enzyme, stimulates immunoglobulin production, tumor suppressor
CAPN1	Down regulation	Cytoplasm, Cell membrane	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
IGF2R	Down regulation	Lysosome membrane	Transport and involved in suppression of breast, tumor and promyelocytic leukemia
GDI2	Down regulation	Cytoplasm, Membrane	Regulate the GDP/GTP exchange reaction
HSPB1	Down regulation	Cytoplasm, Nucleus	Molecular chaperone, stress resistance and actin organization, regulate numerous biological processes
IGHV3-13	Down regulation	Cell membrane	Antigen recognition, immunoglobulins
IGHV1-18	Down regulation	Cell membrane	Antigen recognition, immunoglobulins
LRG1	Down regulation	Vesicles	Transform growth factor beta receptor binding, molecular function and protein binding

Proteins	Regulation	Position	Functions
RHOA	Down regulation	Cell membrane	Regulate a signal transduction pathway, microtubule-dependent signal
PRDX1	Down regulation	Cytoplasm, Melanosome	Participate in cell protection against oxidative stress
G6PD	Down regulation	Cytosol, Microtubule organizing center, Vesicles	Provide reducing power and pentose phosphates for fatty acid and nucleic acid synthesis
BLMH	Down regulation	Cytoplasm	Protect normal and malignant cells from BLM toxicity
TALDO1	Down regulation	Cytoplasm	Balance of metabolites in the pentose-phosphate pathway
DCD	Down regulation	Extracellular	Antimicrobial activity and proteolytic activity
PCSK9	Down regulation	Cytoplasm	Crucial player in the regulation of plasma cholesterol homeostasis and regulate neuronal apoptosis
GAPDH	Down regulation	Cytoplasm, Cytosol, Nucleus	Participate in nuclear events including transcription, RNA transport, DNA replication and apoptosis

The differentially expressed proteins were selected for further analysis. According to the cellular component analysis, the differentially expressed proteins were enriched in extracellular exosomes, followed by cytosol and extracellular spaces (Fig. 2C). These differentially expressed proteins may act on AIS by participating in biological processes such as cell-cell adhesion, proteolysis, and the Fc-epsilon receptor signaling pathway (Fig. 2D). In the AIS patients, some proteins, such as FN1, were upregulated and others, such as VDAC1, RhoA and AHNAK, were downregulated. The FN1, VDAC1, RhoA and AHNAK proteins interacted to influence the development of AIS (Fig. 2E). These results suggest that the plasma exosomal proteins FN1, VDAC1, RhoA and AHNAK are likely biomarkers of AIS.

The RhoA and VDAC1 proteins were downregulated in the AIS patients

To further determine whether the proteins were significantly different in the AIS patients, we selected FN1, RhoA, VDAC1 and AHNAK for verification *in vitro*. Proteomic analysis confirmed that the differentially expressed proteins in the AIS and control groups were mostly distributed in the extracellular exosomes; thus, the characteristics and target proteins of the plasma exosomes were further verified and analyzed in this study.

Extracellular vehicles (EVs) isolated from the plasma of the AIS patients and the controls were identified by using transmission electron microscopy and particle size detection (Fig. 3A, B). Additionally, western blotting was performed to detect the expression of the exosomal marker CD63 and the endoplasmic

reticulum-specific marker calnexin but not the transmembrane protein TSG101 (Fig. 3C). Flow cytometric analysis of the exosomal markers CD63 and CD81 confirmed that the isolated EVs were purified exosomes (Fig. 3D).

To determine whether the RhoA, VDAC1, FN1 and AHNAK protein levels differed between the AIS and control groups, we performed western blot analyses of the two groups. The results showed that the RhoA and VDAC1 protein levels in the AIS group were significantly lower than those in the control group, consistent with the MS results (Fig. 3F). However, the FN1 protein was downregulated in the AIS patients (Fig. 3E), contrary to the MS results. After analyzing the grayscale values, we found that the RhoA protein expression in the controls was greater than twice that of the AIS patients. The AHNAK protein did not differ between the AIS patients and the controls. Taken together, the results showed that RhoA and VDAC1 may play a vital role in AIS patients.

The RhoA and VDAC1 were associated with the severity in the AIS patients

To determine whether RhoA and VDAC1 expression correlates with the severity of AIS patients, we further analyzed the clinical characteristics and protein expression. Comparing the patients' baseline values, we observed that the Cobb angle had a significant association with the RhoA and VDAC1 proteins. The Cobb angle is used for quantitative assessment of the lateral curvature of the spine, representing the severity of AIS. After Pearson analysis, the results showed that the Cobb angle was negatively correlated with the RhoA and VDAC1 proteins (Fig. 4), and the formulas were shown as below:

$$Y_{\text{RHOA}} = -37.71 * X_{\text{cobb}} + 7309 \quad (R^2 = 0.5174)$$

$$Y_{\text{VDAC1}} = -20.27 * X_{\text{cobb}} + 2546 \quad (R^2 = 0.5465)$$

The related pathways were involved in the RhoA and VDAC1 proteins in the AIS patients

To explore how the RhoA and VDAC1 proteins are involved in AIS, we next performed an analysis of the KEGG signaling pathways for *Homo sapiens* (human). The RhoA protein influences AIS primarily through the chemokine signaling pathway, platelet activation and cAMP signaling pathway, and the VDAC1 protein is a key factor that participates in the necroptosis pathway, acting on the development of AIS. These data showed that the RhoA and VDAC1 proteins are likely predictors of AIS. The pathway networks are shown (Fig. 5–6).

Discussion

This is the first report confirming the role of exosomes in AIS. We identified the differentially expressed proteins VDAC1, RhoA, FN1 and AHNAK in AIS, and VDAC1 and RhoA were downregulated, which is

related to the severity and progression of the disease. Exosomes are likely potential biomarkers and predictors of AIS.

Our study first screened the differentially expressed proteins in the total plasma proteins, and the differentially expressed proteins were concentrated in the exosomes. To further identify biomarkers, we chose to verify the proteins in the exosomes. The results demonstrated that the RhoA and VDAC1 proteins were downregulated in AIS, consistent with the MS data. RhoA is an important regulator of platelet function and development^[16]. RhoA directs cytoskeletal reorganization to promote shape changes and particle release during hemostasis, activating^[16] platelets and the signaling pathways of damaged vessel walls. Previous studies have confirmed the elevated levels of calcium and phosphorus in the platelets from AIS patients^[17], and the level of platelet calmodulin is a predictor of progression of the curve^[18], and some proteins over or under expressed by AIS Lenke type 5 patients were involved in the blood coagulation pathway^[21]. In the platelet-skeleton hypothesis, platelet activation is thought to be accompanied by changes in calmodulin in the dilated vessels of AIS, which are related vertebral deformation, while activated platelets release growth factors in the blood vessels of the near-growth plate, stimulating the development of the vertebral endplate after mechanical injury and affecting the progression of AIS^[19]. Therefore, we believe that RhoA affected the scoliosis progression of AIS by regulating platelet function. As a key factor in mitochondrial apoptosis, VDAC1 is involved in the release of mitochondrial proapoptotic proteins into the cytosol, and overexpression and dysregulation of this pathway induce apoptosis and other related diseases. The asymmetry of the paravertebral muscles has been observed in AIS. We hypothesize that under different dynamic cell conditions, the difference between activated chondrocyte proliferation and apoptosis in the vertebral body and the convex side growth plate leads to lateral curvature and rotation. The VDAC1 protein may influence the growth and imbalance of muscles and bones by promoting the apoptosis and proliferation of chondrocytes, resulting in scoliosis. This result is consistent with the previous study, which given that vitamin D binding protein (DBP) is associated with bone metabolism and play an important role in the pathogenesis of AIS or disease severity ^[21].

However, the results for FN1 were inconsistent in MS and western blotting. Most likely, total plasma proteins were mixed with other components detected by MS, but western blotting focused only on the exosomes. Previous studies have shown that FN is increased in AIS patients with Lenke type 5 curvature ^[21]. Therefore, more studies are needed to demonstrate the role of FN1 in AIS in the future, but we believe that exosomes are useful in our study because these particle achieve intercellular material transfer and information exchange and are involved in regulating cellular physiological functions and certain pathological processes^[20].

This study has some limitations. First, the validation of the biomarkers was performed in a small cohort of patients, and only 18 subjects including 10 patients with primary moderate to severe AIS were enrolled. Second, considering that AIS is more common among females than males, this study needed more female subjects. Third, the selection of the target proteins may be biased. Previous studies have reported

that the VDAC1, RhoA, FN1 and AHNAK proteins are related to AIS. Moreover, other related differentially expressed proteins should be studied. Exosomes should be examined in animals in the future.

Conclusions

In this study, the first to examine exosomes in AIS, we demonstrated that exosomes are potential biomarkers of AIS, and RhoA and VDAC1, as target proteins in AIS, were downregulated, predicting the severity of AIS. Therefore, exosomes can probably be used for the early diagnosis of AIS.

Abbreviations

Adolescent idiopathic scoliosis (AIS); Rhodopsin A (RhoA); voltage-dependent anion-selective channel 1 (VDAC1); fibronectin 1 (FN1); mass spectrometry (MS) ; Scoliosis Research Society (SRS); Ingenuity Pathway Analysis (IPA); extracellular vehicles (EVs); vitamin D binding protein (DBP).

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and written informed consent was obtained from each patient.

Consent to publish

The authors have received written consent from participants to publish individual patient data.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' Contributions

QD, NS and XZ participated in the study design, data collection, analysis of data and preparation of the manuscript. LM, NC, XL and YY carried out the experimental work and the data collection. HL, MC, JL participated in the interpretation of data and drafted the manuscript. QD critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

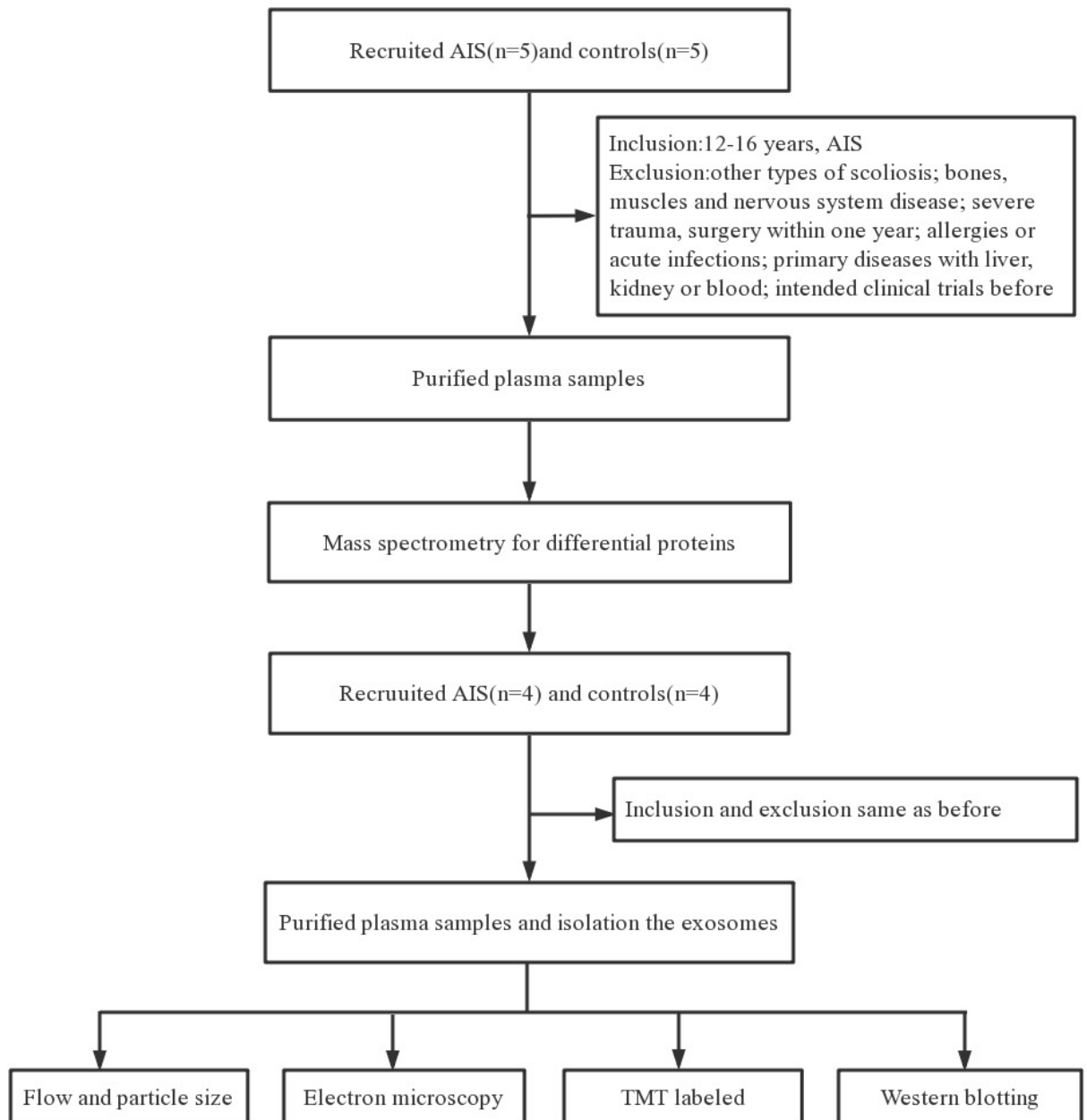


Figure 1

Study enrollment and study processes.

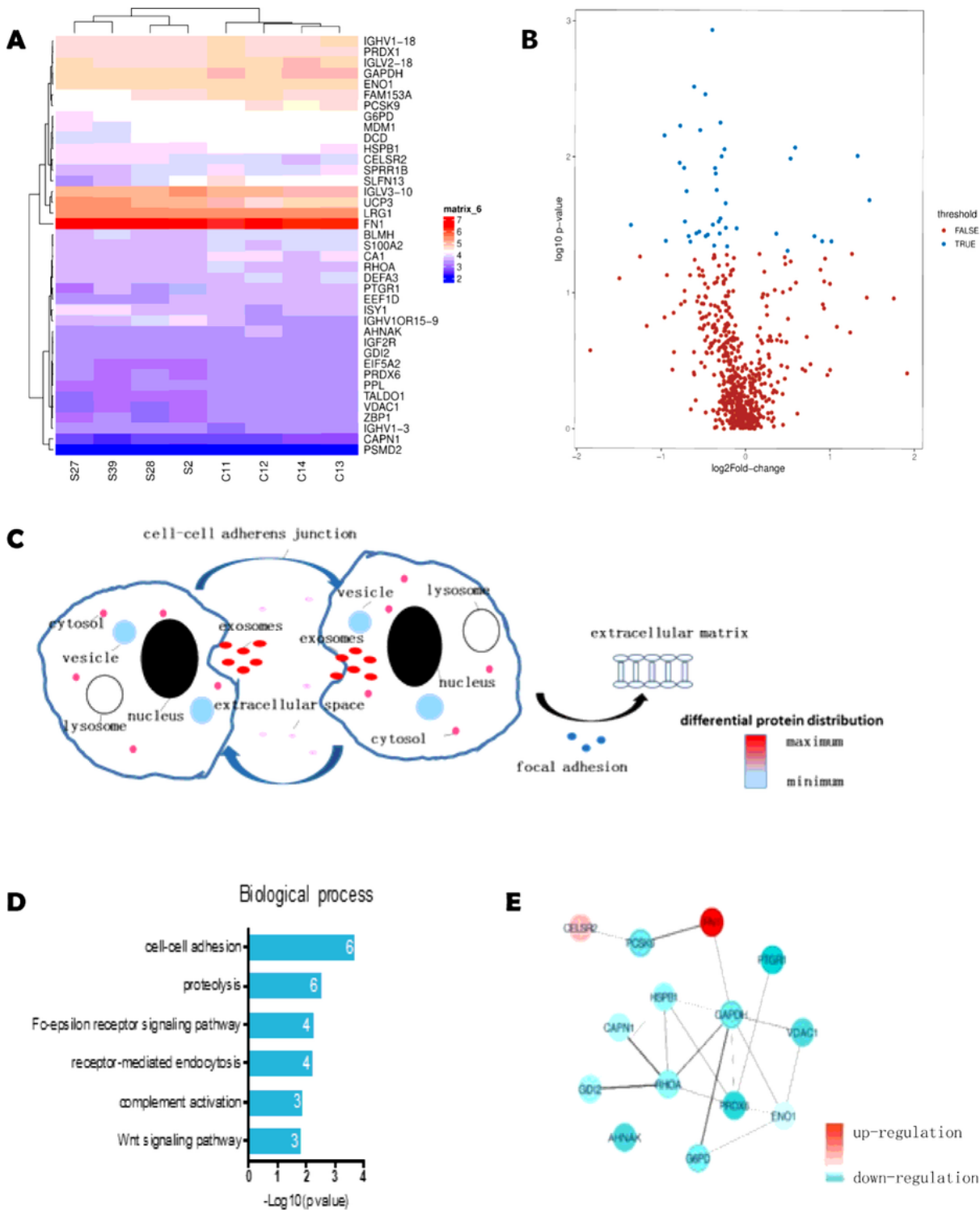


Figure 2

Candidate proteins validated from plasma exosomes. (A) Hierarchical clustering analysis. (B) Volcano plots analysis. (C) Cellular component. (D) Biological process. (E) Differential protein interaction network map.

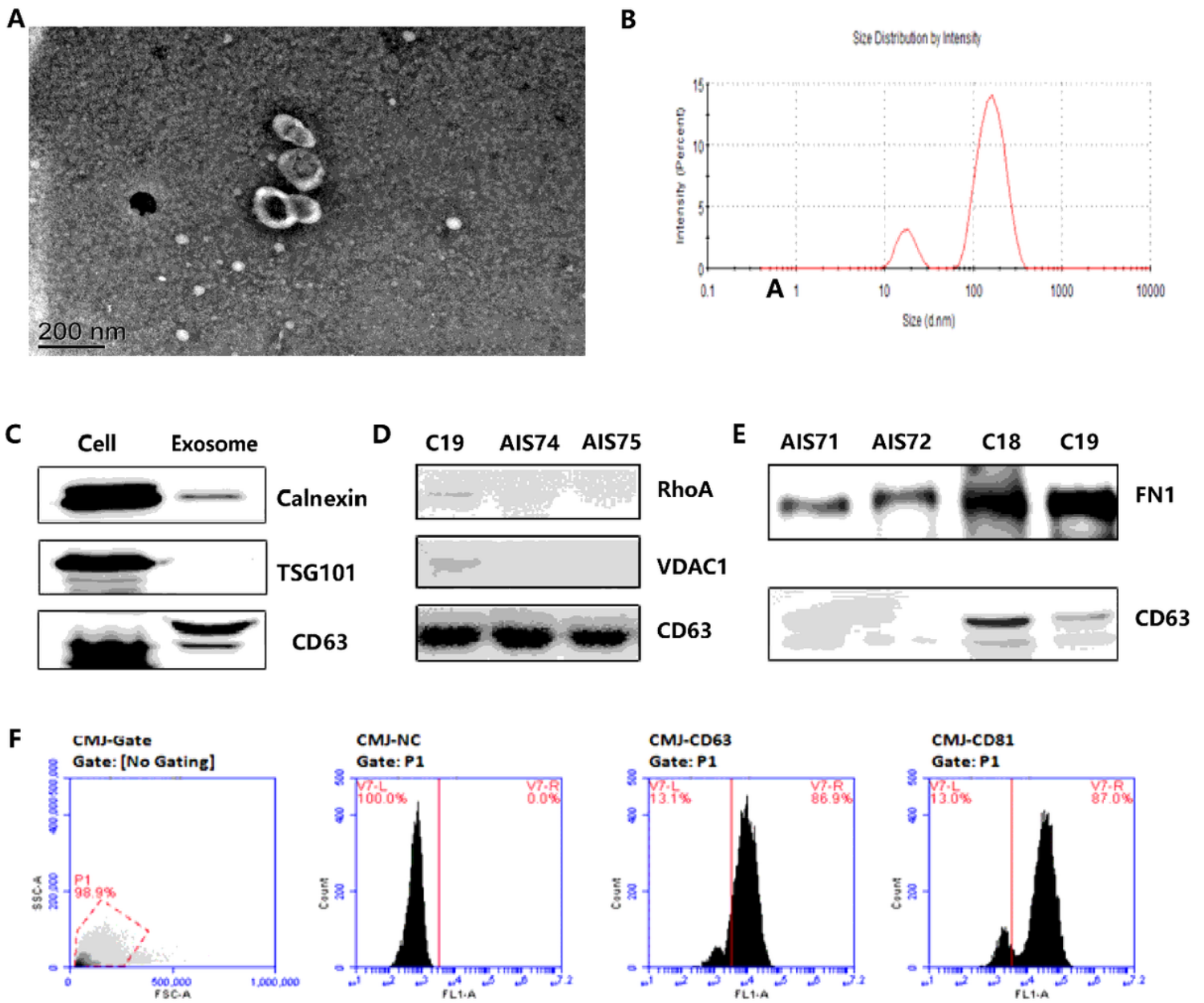


Figure 3

(A) Transmission electron microscope. (B) Particle size. (C) Western blot for exosomal marker. (D) Western blot for RhoA and VDAC1 proteins. (E) Western blot for FN1 proteins. (F) Flow cytometry analysis.

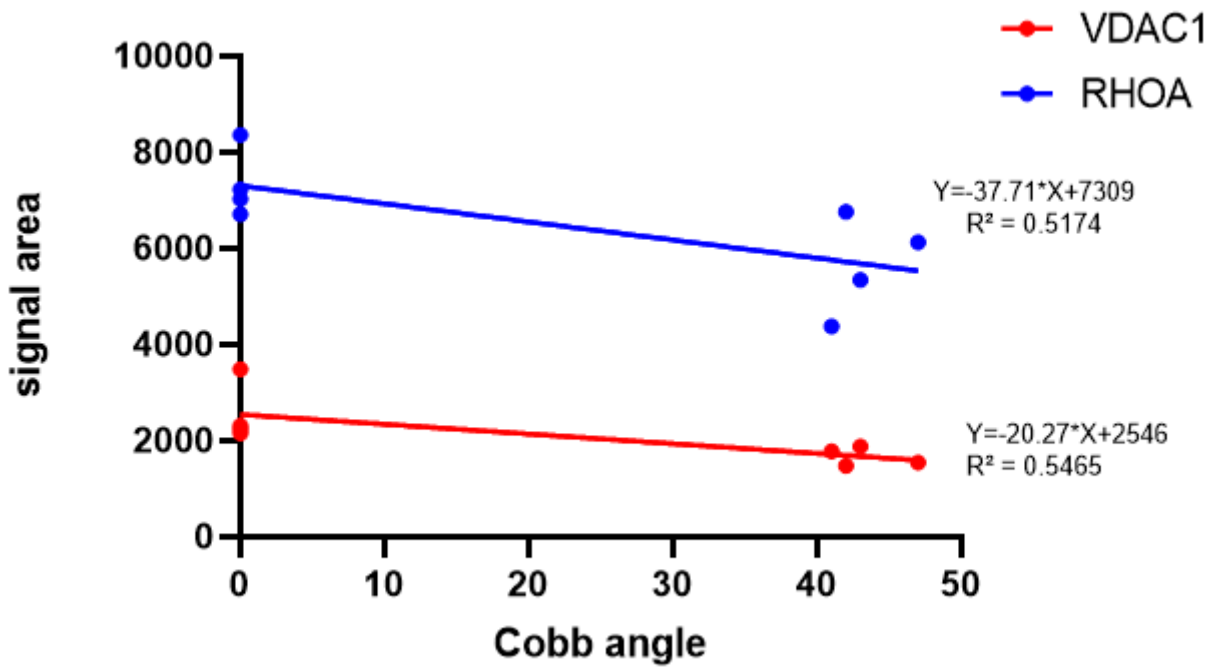


Figure 4

Pearson analysis of VDAC1 and RhoA between normal and AIS.

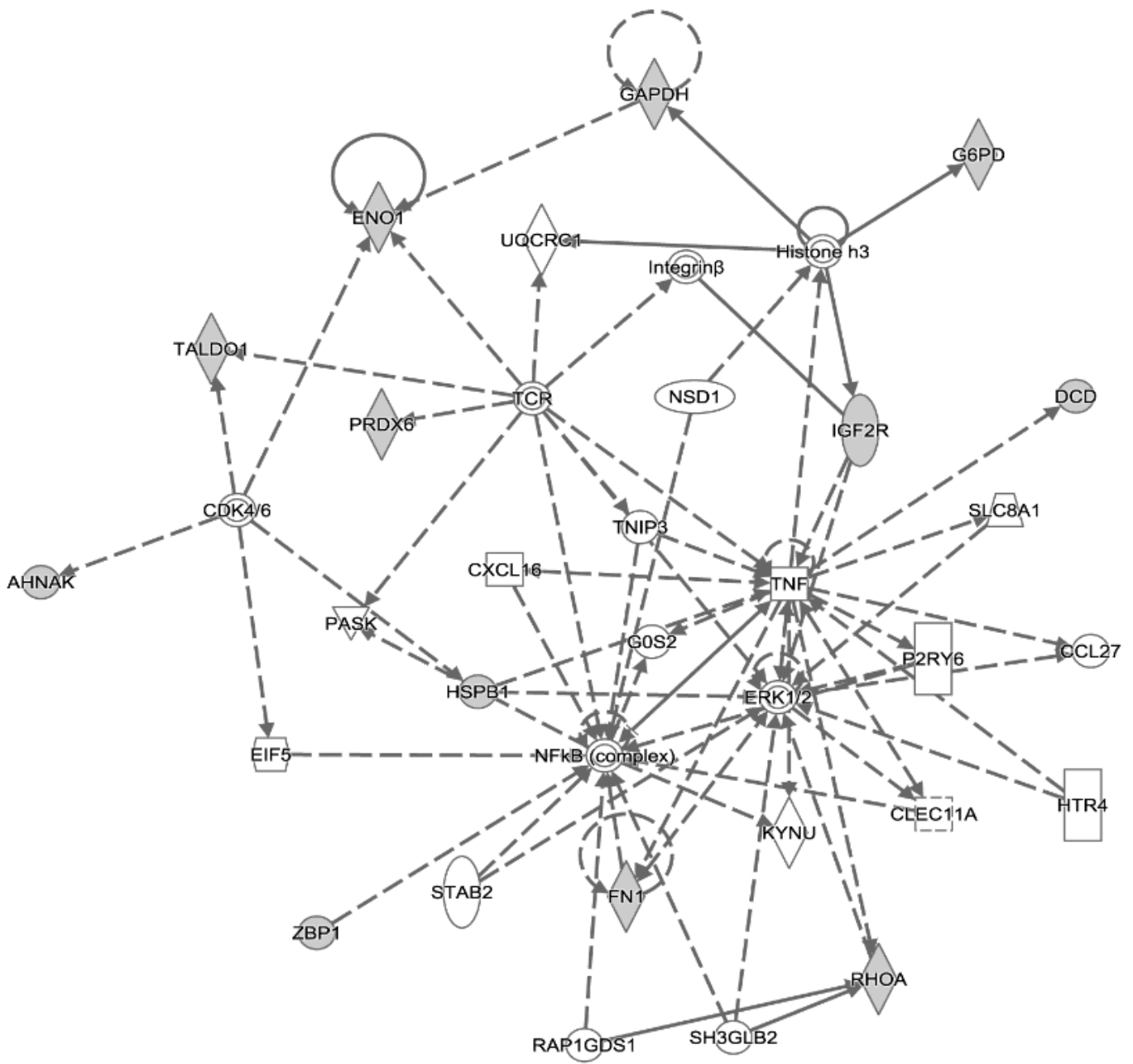


Figure 5

The predicted interactions in the differentially expressed genes related to RhoA, AHNAK and FN1 in the AIS patients.

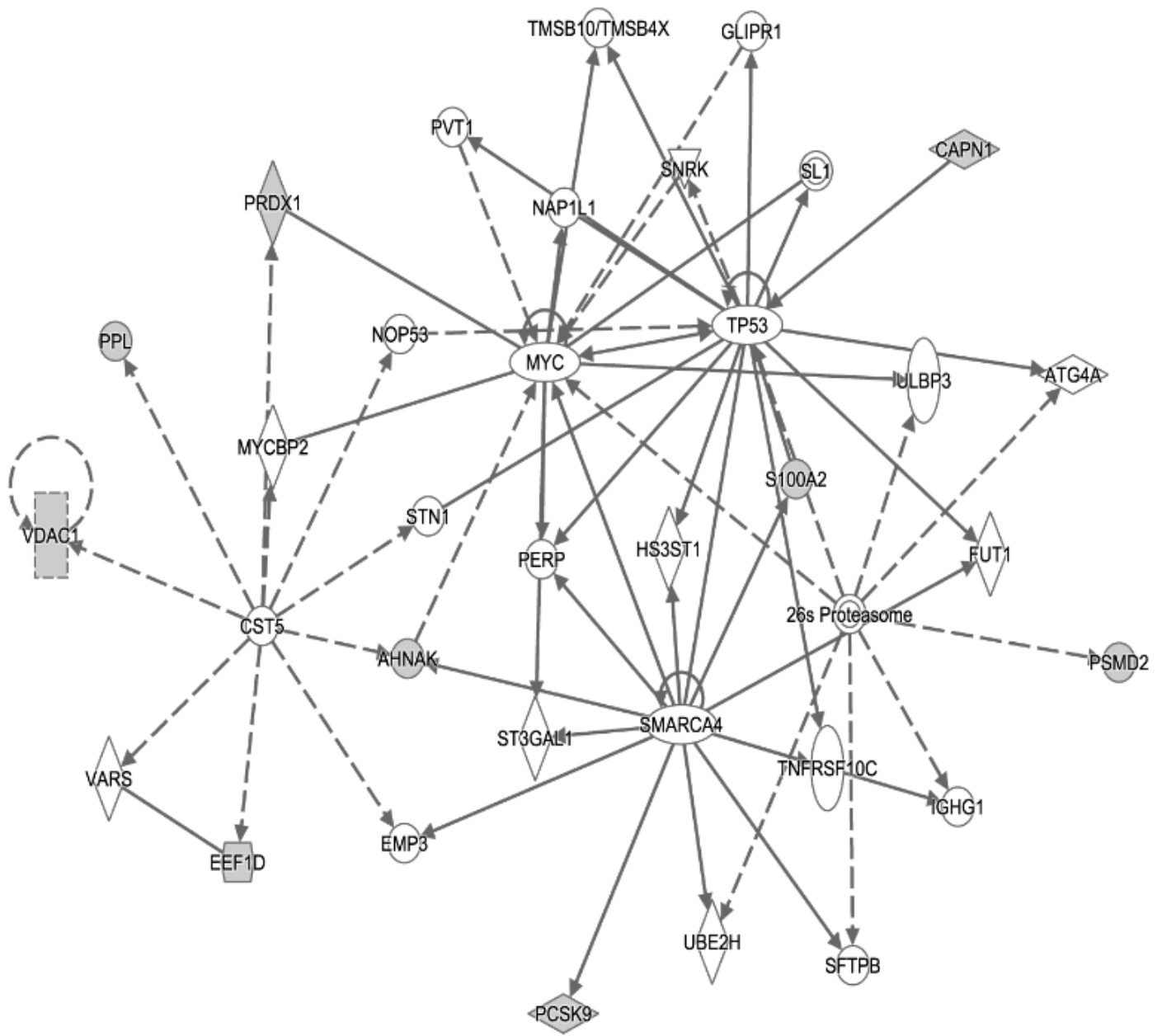


Figure 6

The predicted interactions in the differentially expressed genes related to VDAC1 and AHNAK in the AIS patients.