Research Square

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Plasma exosome proteomics reveals the pathogenesis mechanism of post stroke cognitive impairment

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Abstract

Background

Recently, the plasma exosome biomarkers of post-stroke cognitive impairment (PSCI) have been brought into focus. Exploration and utilization of exosome biomarkers and their related functions provided the possibility for the diagnosis and treatment of PSCI. Aims To identify for new diagnostic and prognostic biomarkers of plasma exosome using label-free quantitative proteomics and biological information analysis in PSCI patients.

Methods

A series of behavioral assessments were performed, including the Mini-Mental Status Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Barthel index, the Morse Fall Seale (MFS) beteen control group (n = 10) and PSCI group (n = 10). The blood samples were collected to analyse the biomarker and differentially expressed proteins of plasma exosome using label-free quantitative proteomics and biological information. The exosomes marker proteins were determined by Western blot. The exosome morphology was observed by transmission electron microscopy.

Results

The scores of MMSE and MoCA were significantly decreasd in the PSCI group. The PT% and high-density lipoprotein decreasd and the INR ratio increasd in PSCI group. The mean size of exosome was approximately 71.6 nm and the concentration was approximately 6.8E + 7 particles/mL. Exosome proteomics identificated 259 differentially expressed proteins, including 131 up-regulated proteins and 128 down-regulated proteins. The mechanisms of cognitive impairment are related to up-regulation of degradation of ubiquitinated proteins, calcium dependent protein binding, cytoskeleton reorganization, platelet aggregation and down-regulation of cell adhesive protein binding, formation of fibrin clot, complement activation, lipid metabolism and ATP-dependent degradation of ubiquitinated proteins in plasma exosome of PSCI patients. Plasma levels of YWHAZ and BAIAP2 were significantly increased while that of IGHD, ABCB6 and HSPD1 were significantly decreased in PSCI patients.

Conclusion

These proteins might be target-related proteins and provide global insights into pathogenesis mechanisms of PSCI at plasma exosome proteins level.

1. Introduction

Ischemic stroke is caused by cerebral artery occlusion, which causes damage to endothelial cells, vascular smooth muscle, glial cells, neurons and related neurovascular units, and ultimately leads to brain tissue death and focal neurological damage(1, 2). Post-stroke cognitive impairment (PSCI) is a type of vascular cognitive impairment that occurs within 6 months after stroke. Patients with stroke lesions occurring in brain regions that are not traditionally cognition-involved may also develop PSCI. The plasma exosome biomarkers of post-stroke cognitive impairment (PSCI) has been brought into focus. Exploration and utilization of plasma exosome biomarkers and their related functions provided the possibility for the diagnosis and treatment of PSCI.

Exosomes are small membrane vesicles derived from endosomes, with a diameter of about 30-100nm. Exosomes exist in extracellular fluid and contain important genetic materials such as DNA, RNA, protein, lipid and miRNA (3). Exosomes can directly or indirectly act on target cells through the release of membrane contents or signal molecules for intercellular information transmission. In different pathological stages of ischemic stroke, exosomes released by different types of nerve cells contain specific signal molecules. Exosome miRNA-122-5p and miR-300-3p were biomarkers for the diagnosis of hyperacute (less than 6h), subacute (8-14d) and convalescent (greater than 14d) ischemic cerebral infarction(4). Exosomes secreted by circulating epcs can transfer their inclusion to recipient endothelial cells, which contain miRNA related to PI3K/Akt signaling pathway and miRNA related to angiogenesis, such as miR-126 and miR-296. In the brain, exosomes secreted by cultured glioma cells provide angiogenic proteins, mRNAs and miRNAs to cerebrovascular endothelial cells and induce angiogenesis(5). Neurons and glial cells interact with exosomes released by them to transfer biomolecules, regulate axonal growth and myelin sheath formation, and participate in brain nerve remodeling. A large number of studies have shown that mesenchymalstem cells (MSCs) derived exosomes can effectively promote vascular and nerve regeneration, reduce inflammatory response and improve traumatic brain injury (TBI)(6, 7). Proteomics is an indispensable "omics" science to elucidate the proteome diversity. Faced with the limitations of diagnosis and treatment of PSCI, the task of finding effective methods to diagnose, predict and prevent PSCI has become more important.

To identify for new diagnostic and prognostic biomarkers of plasma exosome using label-free quantitative proteomics and biological information analysis in PSCI patients.

In this study, a series of behavioral assessments including the Mini-Mental Status Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Barthel index and blood biomarker detection were performed. The plasma exosome from control subjects and PSCI patients were collected and analysed by label-free quantitative proteomics. The biological information analysis of differentially expressed proteins were performed to provide global insights into pathogenesis mechanisms of PSCI at proteins level.

2. Methods

2.1 Study design

This clinical trial had been registered in the Chinese Clinical Trial Registry (ChiCTR) (registration number: ChiCTR1900023739, registration date: June 10, 2019), and the research protocol was approved by the Ethics Committee of Dongzhimen Hospital attached to Beijing University of Chinese Medicine (approval number: DZMEC-KY-2019-04). Patients with acute ischemic stroke were enrolled from Dongzhimen Hospital (eastern area)attached to Beijing University of Chinese Medicine between June 9, 2019 to December, 2019. The study was performed according to the ethical principles of the Helsinki Declaration of 1975 (and as revised in 2013). Written, informed consent was obtained from all participants or their legal guardians. This study contains the control group and PSCI group.

2.2 participants

20 participants were included in this study and signed the informed consent. There are 10 participants in the PSCI group and control group, respectively. PSCI patients' inclusion criteria included the following: (1) age ≥ 35 years and ≤ 70 years; (2) All patients have undergone MRI or CT scan to confirm the acute cerebral infarction; (3) Cognitive evaluation were done by Mini-Mental State Examination (MMSE) within the first 7 days of stroke onset. Patients with a MMSE score ≤ 26 were defined as the PSCI group. (4) Cognitive impairment occurred after stroke. (8, 9). Exclusion criteria included the following: (1) Complicated with severe primary diseases of the heart, liver, kidney and hematopoietic system; (2) Acute disturbance of consciousness; (3) Other causes of dementia and brain dysfunction are ment al and physical disorders; (4) With serious impairment of vision or hearing or speech interferes with rehabilitation; and (5) In addition to cognitive impairment, there were no other focal signs and symptoms o f cerebrovascular disease.

2.3 Behavioral assessment

A series of Behavioral assessments were performed, including the Mini-Mental Status Examination (MMSE), the Montreal Cognitive Assessment (MoCA), the Barthel index, the Morse Fall Seale (MFS), and The Braden Scale for predicting pressure sore risk, and the Padua Prediction Score.

2.4 Blood biomarker detection

The blood samples from an anticoagulated peripheral vein from each patient were collected at the Dongzhimen Hospital of Beijing University of Chinese Medicine. Then, we used the blood samples to measure the fibrinogen, red blood cell specific volume (HCT), total cholesterol (CHO), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and other indicators.

2.5 The isolation and character identification of plasma exosomes

2.5.1 The isolation procedure of exosomes

The isolation of plasma exosomes using TiO2 with a slight modification (10). Remove the plasma sample from storage and place on ice. Centrifuge the plasma sample at 2000 × g for 20 minutes at room temperature to remove cells and debris. Transfer 100 uL plasma to a new tube and processed using 0.2 mm pore size syringe filters (PALL Life Sciences, USA) to remove apoptotic bodies and large microvesicles. Then, the plasma sample was mixed with 5 mg TiO2 microspheres (GL Science Inc, Japan) and mixed the

sample thoroughly by vortexing for 5 min at 4℃. After washing three times with PBS, exosomes were directly lysed and digested with trypsin (Promega, Madison, WI) from the surface of microspheres in 50 mM ammonium bicarbonate at 37°C for 16h. The supernatant were transferred to a new tube and the TiO2 microspheres were washed twice with 100μLof 0.1% FA following centrifugation at 12,000 g for 5 min at 25°C. The washing fraction were collected and pooled with the supernatant. The peptide concentration was measured using NanoDrop (Thermo Fisher, America) at an absorbance of 280 nm.

2.5.2 Western blot analysis of exosomes marker proteins

Detect the quality of plasma protein in 0.5 mL fraction sample. The fraction of vesicles with the least plasma protein content was selected for subsequent analysis. The protein concentration of the sample was determined by the BCA method. 10 μg of the protein was separated by 12% SDS-PAGE and transferred to a 0.45 μm PVDF membrane, and blocked with a blocking solution containing 5% bovine serum albumin for 1 h at room temperature. Exosomal marker protein antibodies (TSG101, CD9, CD63, CD81) were add respectively, and incubate overnight at 4°C. After being eluted with 1×TBST buffer, secondary antibody was add and incubate for 90 min at room temperature. After washing 3 times with TBST, the color was developed using SuperSignal West Femto Substrated Trial Kit (34094, Piece, USA).

2.5.3 Transmission electron microscopy (TEM)

TEM was utilized to observe the exosome morphology. Exosome sample drops were allowed to adsorb to formvar-coated EM grids for 5 min, and were negatively stained with 2% (w/v) phosphotungstic acid for 1 min. TEM analysis was performed at an acceleration voltage of 80 kV with a transmission electron microscope (H-7650; Hitachi, Ltd., Tokyo, Japan).

2.5.4 Dynamic light scattering (DLS) analysis and Nanoparticle tracking analyzer (NTA)

The particle size and concentration analysis of model exosomes were performed on ZetaView Nanoparticle Tracker (Particle Metrix, Meerbusch, Germany). Calibrate the instrument with polystyrene particles with a particle size of about 100 nm, Dilute the model exosomes to approximately 1×10^8 particles/mL, and put them into the instrument for analysis. Each group of samples is automatically scanned 11 times to remove abnormal data. The data was recorded and analyzed by ZetaView 8.03.04.01 software.

2.6 Label-free quantitative proteomics

The proteins were identification by MALDI-TOF-MS/MS and database searching. An online liquid chromatography-tandem mass spectrometry (LC-MS/MS) setup consisting of an EasynanoLC system and a Q-Exactive mass spectrometer (Thermo Scientific,Germany) equipped with a nanoelectrospray ion source was used for all LC-MS/MS experiments.

1) Mobile phase A consisted of 0.1% FA, 2% acetonitrile in water, and mobile phase B consisted of 0.1% FA, 98% acetonitrile in water. The flow rate is 300 nL/min.

2)The source was operated at 2 kV. For full MS survey scan, AGC target was 3e6, scan range was from m/z 300 to 1400 with the resolution of 70,000. The 50 most intense peaks with charge state 2 and above were selected for sequencing and fragmented in the ion trap by HCD with normalized collision energy of 27%. Exclude isotope item was on and dynamic exclusion time was set as 18s.

3) Raw MS files were searched against uniprot database using MaxQuant software. (version 1.5.2.8). The fixed modification was C (carbamidomethyl) and the variable modification was M (oxidation) and protein N-term (acetyl). The first search peptide tolerance was 20ppm and the main search peptide tolerance was 6ppm. The MS/MS tolerance was 0.02Da. The false discovery level in PSM and protein was 1%. Match between runs was used and minimum score for modified peptides was 40.

2.7 Biological information analysis

2.7.2 Protein-protein interaction (PPI) networks analysis

To better understanding of the biological context of the differentially expressed proteins, protein interaction analysis was carried out with the free web-based search tool STRING10.5. STRING is one of ELIXIR's core data resources and is a database of known and predicted protein interactions. The STRING database aims to collect and integrate this information, by consolidating known and predicted protein-protein association data for a large number of organisms(11). STRING was required to add more predicted functional partners to facilitate the creation of PPI networks. The list of differentially expressed protein IDs were input into the STRING database (https://string-db.org) to identify known and predicted protein functional association networks.

2.7.1 GO analysis

To characterize the differentially expressed proteins, a GO enrichment analysis was performed to classify the proteins according to their protein classification, biological process, cellular component, and pathway. In order to obtain the distribution of these experimentally identified proteins, GO enrichment annotation tools were employ to note each protein with high throughput and to grasp the correlation between protein and biological function in a whole. GO function enrichment analysis of protein, refers to the use of this distribution was compared with the distribution of the overall protein, confirming experimentally identified proteins were significantly enriched in which categories biological processes or molecular functions, There are a lot of metabolic, regulatory and signal transduction processes resisting in the organism, and these processes often form several different pathways. Pathway analysis allows us to identify the most important biochemical- metabolic pathways and signal transduction pathways in which the proteins were involved.

2.8 ELISA quantification

To detect the levels of IGHD, ABCB6, HSPD1, YWHAZ and BAIAP2, the plasma were determined by enzyme-linked immunosorbent assay (ELISA) kits. According to the instructions of the manufacturer, plasma samples were thawed and submitted to ELISA quantitative analysis using human IgD ELISA kit (Abcam, ab157708, Cambridge, UK), human ATP Binding Cassette Subfamily B Member 6, Mitochondrial (ABCB6)

ELISA Kit (Abbexa, abx385541, Cambridge, UK), human Heat Shock Protein 60, HSP-60 ELISA Kit (CSB-E08560h, Cusabio, China), human 14-3-3 protein zeta/delta (YWHAZ) ELISA kit (CSB-EL026293HU, Cusabio, China), human Brain-Specific Angiogenesis Inhibitor 1-Associated Protein 2 (BAIAP2) ELISA Kit (Abbexa, abx386001, Cambridge, UK). ELISA microplates were read using MK3 microplate reader (Thermo, Helsinki, Finland).

2.9 Statistical analysis

All values were shown as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS20.0. The Demographic data and MMSE, MoCA, blood marker were analyzed with independent-samples t test between control group and PSCI group. Chi-square test was used to analyze gender difference between two groups. P values < 0.05 were considered statistically significant.

3. Results

3.1 Demographics, behavioral assessment and blood biomarker detection results

There were no differences in age (P = 0.182), gender (P = 0.653) or education (P = 0.072) between the PSCI and the control groups. The scores of MMSE and MoCA were significantly decreasd in PSCI groups (P < 0.01) (Table 1). Compared with control group, the PT% and high-density lipoprotein decreasd (P < 0.05) and the INR ratio increasd (P < 0.05) in the PSCI group (Table 2).

Table 1
Demographic information and behavioral scale assessment

	Control group (n = 10)	PSCI group (n = 10)	P value
Age (y)	55.80 ± 6.92	60.30 ± 9.17	0.231
Gender (M/F)	6/4	5/5	0.653
Education (y)	10.30 ± 2.16	8.30 ± 2.50	0.072
MMSE	29.80 ± 0.42	20.30 ± 3.83	0.01
MoCA	29.40 ± 0.70	15.70 ± 5.03	0.01
Barthel	—	70.00 ± 15.28	—
Morse Fall Seale	—	46.50 ± 10.81	—
Braden Scale	—	20.50 ± 2.32	—
Padua Prediction Score	—	2.70 ± 2.06	—

Note: MMSE, Mini-Mental Status Examination; MoCA, Montreal Cognitive Assessment; Barthel: the Barthel index, and data are presented as mean ± SD or number of patients.

Table 2
The blood marker detection results

	Control group (n = 10)	PSCI group (n = 10)	P value
Blood Coagulation function			
PT%	102.13 ± 9.67	91.90 ± 7.58	0.027*
INR ratio	1.00 ± 0.05	1.06 ± 0.06	0.045*
Activated partial thromboplastin time	30.14 ± 3.99	31.33 ± 5.19	0.619
Fibrinogen content	2.61 ± 0.26	2.80 ± 0.62	0.470
thrombin time	15.37 ± 1.16	14.86 ± 1.33	0.424
D-dimer	62.43 ± 27.65	118.6 ± 73.42	0.075
Blood biochemistry			
Serum creatinine	61.40 ± 14.89	60.60 ± 21.26	0.923
Glucose	6.54 ± 2.35	8.87 ± 3.68	0.109
Albumin	39.07 ± 4.57	38.44 ± 4.52	0.760
calcium	2.29 ± 0.09	2.34 ± 0.13	0.368
Total bilirubin	13.69 ± 3.55	19.93 ± 8.57	0.047
Direct bilirubin	3.23 ± 1.10	4.54 ± 2.47	0.143
Indirect bilirubin	10.46 ± 3.32	15.39 ± 6.43	0.045
Alanine aminotransferase	17.30 ± 6.45	24.90 ± 13.16	0.118
Aspartate aminotransferase	19.40 ± 4.27	34.40 ± 35.08	0.196
Lactate dehydrogenase	152.67 ± 29.82	210.90 ± 47.47	0.006
hydroxybutyric acid dehydrogenase	114.33 ± 22.87	154.50 ± 40.21	0.017
γ-glutamyltransferase	17.70 ± 6.63	29.50 ± 18.85	0.078
alkaline phosphatase	60.89 ± 8.62	69.70 ± 20.31	0.245
Adenylate dehydrogenase	10.00 ± 2.87	10.70 ± 5.44	0.735
Serum lipid			
total cholesterol	4.38 ± 0.56	4.18 ± 1.46	0.691
Apolipoprotein A1	1.17 ± 0.11	1.10 ± 0.17	0.401
Apolipoprotein B	0.83 ± 0.23	0.74 ± 0.26	0.501

Note: Values are mean ± SD.

	Control group (n = 10)	PSCI group (n = 10)	P value
high-density lipoprotein	0.98 ± 0.15	0.84 ± 0.13	0.045*
low density lipoprotein	2.62 ± 0.55	2.42 ± 1.25	0.662
Lipoprotein (a)	182.00 ± 115.42	267.78 ± 176.09	0.285
Homocysteine	13.63 ± 3.75	15.78 ± 7.62	0.512
Note: Values are mean ± SD.			

3.2 Isolation and characterization resuits of plasma exosomes

The content detection results of plasma protein in 0.5 mL fraction sample were shown in Fig. 1A. The content of plasma protein in fractions 6–10 is relatively lower, and the purity of exosomes is relatively high. Therefore, the 5 vesicle fractions 6–10 were selected for subsequent analysis. Western blot analysis was conducted to detect the exosomal protein markers. The protein expression levels of exosomal markers including TSG101 CD9, CD63, CD81 were shown in Fig. 1B. The exosome morphology and size were visualized by TEM (Fig. 1C). TEM observation showed a very homogenous exosome mixture with a typical round morphology with a diameter range of 30–200 nm. The content of plasma protein in 0.5 mL fraction sample is shown in Fig. 1A. The content of plasma protein in fractions 6–10 is relatively small, and the purity of exosomes is relatively high. Therefore, the 5 vesicle fractions 6–10 were selected for subsequent analysis. Western blot analysis was conducted to detect the exosomal protein markers. As shown in Fig. 1B. As exosomal protein markers, CD9, CD63, CD81, TSG101 were were detected in all the plasma exosome samples. NTA was exploited to measure the size distribution of particles and view the isolated vesicle samples (Fig. 1E-F). The fluorescent detection of the samples analyzed in the scatter mode. The size is calculated by the diffusion behavior. The mean size was approximately 71.6 nm and the concentration was approximately 6.8E + 7 particles/mL.

3.3 Identification of the differentially expressed exosome proteins between the control group and PSCI group

In total, 259 differentially expressed exosome proteins were quantified and identified between the control group and PSCI group by Label-free quantitative proteomics, of which 131 proteins showed up-regulated expression and 128 proteins showed down-regulated expression. The differentially expressed proteins with ratios PSCI/control higher or lower than 1.2-fold change with adjusted P value < 0.05 were considered to be significantly changed. The volcano plot of differentially expressed proteins were shown in Fig. 2. The heat map of all differentially expressed proteins were shown in Fig. 3. The identification results of main 30 up-regulated proteins were shown in Table 3 and the other 101 up-regulated proteins were shown in Table S1. The identification results of main 30 down-regulated proteins were shown in Table 4 and the other 98 down-regulated proteins were shown in Table S2. The detailed results of these proteins are summarized. As

shown in Table 5, the molecular function and biological process category of 30 up-regulated proteins were performed based on biological function. The molecular function and biological process category of 27 down-regulated proteins were shown in Table 6.

Table 3

The identification results of main 30 up-regulated proteins between the PSCI group and control group

NO.	Accession NO.	Protein name	Symbol	Exp. Mr (KDa)	Protein score	Fold Change (PSCI/ control)	P value
1	P63104	14-3-3 protein zeta/delta	YWHAZ	27.75	177.81	2.13	0.001
2	P31947	14-3-3 protein sigma	SFN	27.77	8.19	3.04	0.01
3	Q9UQB8	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	60.87	43.47	4.95	0.01
4	P78417	Glutathione S-transferase omega-1	GSTO1	25.90	27.57	3.00	0.01
5	P61421	V-type proton ATPase subunit d 1	ATP6V0D1	40.33	8.42	2.91	0.01
6	P34932	Heat shock 70 kDa protein 4	HSPA4	94.33	7.37	7.05	0.01
7	P40197	Platelet glycoprotein V	GP5	60.96	49.41	5.19	0.01
8	P37235	Hippocalcin-like protein 1	HPCAL1	22.31	5.46	5.82	
9	Q9Y6B6	GTP-binding protein SAR1b	SAR1B	22.41	5.08	2.28	0.01
10	P54920	Alpha-soluble NSF attachment protein	NAPA	17.26	33.23	34.53	0.01
11	P13746	class I histocompatibility antigen, A-11 alpha chain	HLA-A	15.44	40.94	33.79	0.05
12	P51575	P2X purinoceptor 1	P2RX1	13.33	44.98	15.11	0.01
13	O76074	cGMP-specific 3,5-cyclic phosphodiesterase	PDE5A	17.60	99.98	11.74	0.01
14	Q13642	Four and a half LIM domains protein 1	FHL1	16.00	36.26	8.72	0.01
15	Q9BQE5	Apolipoprotein L2	APOL2	2.9676	37.092	5.85	0.01
16	P08648	Integrin alpha-5	ITGA5	10.36	114.54	5.73	0.01
17	O94804	Serine/threonine-protein kinase 10	STK10	11.78	112.13	3.86	0.01
18	P11169	Solute carrier family 2, facilitated glucose transporter member 3	SLC2A3	76.41	53.92	3.65	0.01

Note: In the title line, Exp. Mr represented the experimental molecular weight of the proteins.

NO.	Accession NO.	Protein name	Symbol	Exp. Mr (KDa)	Protein score	Fold Change (PSCI/ control)	P value
19	Q15762	CD226 antigen	CD226	23.75	38.61	3.45	0.01
20	Q93084	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	ATP2A3	17.16	113.98	3.04	0.01
21	O00194	Ras-related protein Rab-27B	RAB27B	21.19	24.61	2.69	0.01
22	Q9NP79	Vacuolar protein sorting-associated protein VTA1 homolog	VTA1	28.89	33.88	2.63	0.01
23	P14770	Platelet glycoprotein IX	GP9	71.37	19.05	2.61	0.01
24	Q9UN37	Vacuolar protein sorting-associated protein 4A	VPS4A	11.56	48.897	2.37	0.001
25	Q08830	Fibrinogen-like protein 1	FGL1	2.9253	36.379	9.61	0.001
26	P02763	Alpha-1-acid glycoprotein 1	ORM1	13.49	23.51	2.12	0.01
27	P02749	Beta-2-glycoprotein 1	APOH	233.67	38.30	2.11	0.01
28	P55058	Phospholipid transfer protein	PLTP	20.08	54.74	2.10	0.01
29	P30273	High affinity immunoglobulin epsilon receptor subunit gamma	FCER1G	10.82	9.67	2.04	0.01
30	P01624	Ig kappa chain V-III region POM	IGKV3-15	17.61	11.92	2.03	0.01

Note: In the title line, Exp. Mr represented the experimental molecular weight of the proteins.

Table 4

The identification results of main 30 down-regulated proteins between the PSCI group and control group

NO.	Accession NO.	Target protein	Symbol	Exp. Mr (KDa)	Protein score	Fold change (PSCI/ control)	P value
1	P06276	Cholinesterase	BCHE	12.41	68.42	0.41	0.05
2	P01880	Ig delta chain C region	IGHD	42.25	17.46	0.02	0.01
3	P04438	Ig heavy chain V-II region SESS		16.32	4.15	0.18	0.01
4	P01771	Ig heavy chain V-III region HIL		13.44	39.48	0.27	0.01
5	P01778	Ig heavy chain V-III region ZAP		12.34	6.03	0.10	0.01
6	P07357	Complement component C8 alpha chain	C8A	65.16	22.61	0.14	0.01
7	P20618	Proteasome subunit beta type-1	PSMB1	26.49	12.65	0.11	0.01
8	Q9P289	Serine/threonine-protein kinase 26	STK26	46.53	2.66	0.13	0.01
9	Q99536	Synaptic vesicle membrane protein VAT-1 homolog	VAT1	8.1909	41.92	0.48	0.01
10	P03952	Plasma kallikrein	KLKB1	71.37	7.30	0.20	0.01
11	P53396	ATP-citrate synthase	ACLY	120.84	21.57	0.41	0.01
12	Q9NP58	ATP-binding cassette sub-family B member 6, mitochondrial	ABCB6	93.88	4.74	0.24	0.01
13	P62330	ADP-ribosylation factor 6	ARF6	20.08	11.40	0.26	0.05
14	P10809	60 kDa heat shock protein, mitochondrial	HSPD1	61.05	88.42	0.47	0.01
15	O15162	Phospholipid scramblase 1	PLSCR1	35.05	10.23	0.11	0.01
16	Q99828	Calcium and integrin-binding protein 1	CIB1	21.70	8.13	0.11	0.01
17	Q5D862	Filaggrin-2	FLG2	248.07	81.40	0.12	0.01
18	Q8WXI7	Mucin-16	MUC16	2284.30	8.38	0.13	0.01

Note: In the title line, Exp. Mr represented the experimental molecular weight of the proteins.

NO.	Accession NO.	Target protein	Symbol	Exp. Mr (KDa)	Protein score	Fold change (PSCI/ control)	P value
19	P56199	Integrin alpha-1	CFHR1	130.85	4.11	0.14	0.01
20	P25311	Zinc-alpha-2-glycoprotein	AZGP1	34.26	8.18	0.15	0.01
21	P02545	Prelamin-A/C	LMNA	74.14	26.39	0.01	0.01
22	P30486	class I histocompatibility antigen, B-48 alpha chain	HLA-B	40.36	29.55	0.03	0.01
23	Q9NP59	Solute carrier family 40 member 1	SLC40A1	62.54	9.51	0.10	0.01
24	P13164	Interferon-induced transmembrane protein 1	IFITM1	13.96	6.85	0.10	0.01
25	O14818	Proteasome subunit alpha type-7	PSMA7	27.89	17.47	0.17	0.01
26	P22234	Multifunctional protein ADE2	PAICS	47.08	10.28	0.17	0.01
27	P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	65.31	10.92	0.18	0.01
28	Q9HCM2	Plexin-A4	PLXNA4	212.45	5.19	0.18	0.05
29	Q8NG06	E3 ubiquitin-protein ligase TRIM58	TRIM58	54.77	29.55	0.18	0.05
30	Q9BS26	Endoplasmic reticulum resident protein 44	ERP44	46.97	3.45	0.19	0.01

Note: In the title line, Exp. Mr represented the experimental molecular weight of the proteins.

Table 5

The molecular function and biological process category of main 30 up-regulated expressed proteins in PSCI

NO.	Target protein	Molecular function	Biological process
1	14-3-3 protein zeta/delta	cadherin binding; ion channel binding; protein domain specific binding; protein kinase binding	establishment of Golgi localization; protein insertion into mitochondrial membrane involved in apoptotic signaling pathway; synaptic target recognition
2	14-3-3 protein sigma	cadherin and phosphoprotein binding; protein kinase C inhibitor activity	intrinsic apoptotic signaling pathway; regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway; release of cytochrome c from mitochondria
3	Brain-specific angiogenesis inhibitor 1-associated protein 2	cadherin binding involved in cell-cell adhesion; identical protein binding; proline-rich region binding; scaffold protein binding ; transcription cofactor binding	axonogenesis; cellular response to L-glutamate; modification of synaptic structure, modulating synaptic transmission; regulation of synaptic plasticity; vascular endothelial growth factor receptor signaling pathway
4	Glutathione S-transferase omega-1	glutathione dehydrogenase (ascorbate) activity; glutathione transferase activity; methylarsonate reductase activity; oxidoreductase activity	L-ascorbic acid metabolic process; cellular response to arsenic-containing substance; glutathione derivative biosynthetic process; interleukin-12 mediated signaling pathway
5	V-type proton ATPase subunit d 1	proton-exporting ATPase activity, phosphorylative mechanism proton-transporting ATPase activity	IRE1-mediated unfolded protein response; cellular iron ion homeostasis; cellular response to increased oxygen levels; phagosome acidification; proton transmembrane transport
6	Heat shock 70 kDa protein 4	ATP binding	chaperone-mediated protein complex assembly; protein insertion into mitochondrial outer membrane; response to unfolded protein
7	Platelet glycoprotein V	mediates vWF-dependent platelet adhesion to blood vessels	blood coagulation, intrinsic pathway; cell adhesion; platelet activation
8	Hippocalcin-like protein 1	calcium ion binding	

NO.	Target protein	Molecular function	Biological process
9	GTP-binding protein SAR1b	GTP binding; GTPase activity; metal ion binding	antigen processing and presentation of exogenous peptide antigen via MHC class I and II; endoplasmic reticulum to Golgi vesicle-mediated transport; intracellular protein transport;
10	Alpha-soluble NSF attachment protein	Protein containing complex binding; soluble NSF attachment protein activity; syntaxin binding	endoplasmic reticulum to Golgi vesicle mediated transport; synaptic vesicle priming; synaptic transmission, glutamatergic
11	P2X purinoceptor 1	ATP binding; ATP-gated ion channel activity; extracellularly ATP-gated cation channel activity; purinergic nucleotide receptor activity; zinc ion binding	apoptotic process; calcium ion transport; neuronal action potential; regulation of presynaptic cytosolic calcium ion concentration; synaptic transmission
12	cGMP-specific 3,5-cyclic phosphodiesterase	3',5'-cyclic-GMP phosphodiesterase activity; cGMP binding; metal ion binding	cGMP catabolic process; MAP kinase activity; regulation of nitric oxide mediated signal transduction
13	Four and a half LIM domains protein 1	ion channel binding;metal ion binding	cell differentiation; potassium ion transport; membrane depolarization; regulation of potassium ion transmembrane transporter activity
14	Apolipoprotein L2	High density lipoprotein particle binding; lipid binding; signaling receptor binding	cholesterol metabolic process; lipid metabolic process; lipid transport; lipoprotein metabolic process
15	Tetraspanin-32	cytoskeleton organization	cell-cell signaling; defense response to protozoan; integrin mediated signaling pathway; platelet aggregation;
16	Integrin alpha-5	epidermal growth factor receptor binding; metal ion binding; platelet derived growth factor receptor binding; vascular endothelial growth factor receptor 2 binding	angiogenesis; cell adhesion; cell substrate junction assembly; endodermal cell differentiation; cell migration; peptidyl tyrosine phosphorylation;
17	Serine/threonine-protein kinase 10	ATP binding;identical protein binding; protein homodimerization activity; protein serine/threonine kinase activity	activation of protein kinase activity; lymphocyte aggregation and migration; neutrophil degranulation; protein autophosphorylation;

NO.	Target protein	Molecular function	Biological process
18	Solute carrier family 2, facilitated glucose transporter member 3	glucose binding;glucose transmembrane transporter activity	L-ascorbic acid metabolic process; carbohydrate metabolic process; glucose transmembrane transport; neutrophil degranulation
19	CD226 antigen	cell adhesion molecule binding; integrin binding; protein kinase binding	cell recognition; cytokine production; T cell receptor signaling pathway; immunoglobulin mediated immune response; interferon-gamma production
20	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	ATP binding; calcium transmembrane transporter activity; metal ion binding; proton exporting ATPase activity	calcium ion transmembrane transport; calcium ion transport; cellular calcium ion homeostasis; ion transmembrane transport
21	Ras-related protein Rab-27B	GDP binding; GTP binding; GTPase activity; myosin V binding; protein domain specific binding	Rab protein signal transduction; anterograde axonal protein transport; intracellular protein transport; multivesicular body sorting pathway; synaptic vesicle endocytosis
22	Vacuolar protein sorting-associated protein VTA1 homolog	protein C-terminus binding	ESCRT III complex disassembly; endosomal transport; macroautophagy; multivesicular body assembly; multivesicular body sorting pathway
23	Platelet glycoprotein IX	platelet activation apparently involves disruption of the macromolecular complex of GP-Ib with the platelet glycoprotein IX	blood coagulation, intrinsic pathway; cell adhesion; platelet activation
24	Vacuolar protein sorting-associated protein 4A	ATP binding; ATPase activity; protein C-terminus binding; protein domain specific binding; protein containing complex binding	ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway; exosomal secretion; multivesicular body assembly
25	Fibrinogen-like protein 1	inhibiting inflammatory immune responses and metabolic function	adaptive immune response
26	Alpha-1-acid glycoprotein 1	Functions as transport protein in the blood stream	inflammatory response; neutrophil degranulation ; platelet degranulation; interleukin-1 beta secretion

NO.	Target protein	Molecular function	Biological process
27	Beta-2-glycoprotein 1	heparin binding; lipid binding; lipoprotein lipase activator activity; phospholipid binding	angiogenesis; plasminogen activation; platelet degranulation; blood coagulation; regulation of fibrinolysis; triglyceride metabolic process
28	Phospholipid transfer protein	ceramide binding and transfer activity; lipid transporter activity; phosphatidic acid binding and transfer activity;	ceramide transport; high-density lipoprotein particle remodeling ; lipid metabolic process; phospholipid transport; cholesterol efflux; vitamin E biosynthetic process
29	High affinity immunoglobulin epsilon receptor subunit gamma	IgE binding, IgE receptor activity, IgG binding, protein homodimerization activity	immunoglobulin mediated immune response, innate immune response, neutrophil chemotaxis, T cell differentiation
30	Ig kappa chain V-III region POM	antigen binding	Fc-gamma receptor signaling pathway involved in phagocytosis; complement activation, classical pathway; immune response; leukocyte migration;

Table 6
The molecular function and biological process category of main 27 down-regulated proteins in PSCI

NO.	Target protein	Molecular function	Biological process
1	Cholinesterase	acetylcholinesteraseactivity; amyloid-beta binding; choline binding; cholinesterase activity; enzyme binding	choline metabolic process; cocaine metabolic process; neuroblast differentiation; response to alkaloid and folic acid; response to glucocorticoid
2	Ig delta chain C region	antigen binding; immunoglobulin receptor binding	B cell receptor signaling pathway; complement activation, classical pathway; innate immune response; positive regulation of interleukin-1 secretion
3	Complement component C8 alpha chain	complement binding; protein-containing complex binding	complement activation, alternative or classical pathway; immune response; regulation of complement activation
4	Proteasome subunit beta type-1	endopeptidase activity; threonine type endopeptidase activity	proteasomal ubiquitin dependent protein catabolic process; Wnt signaling pathway; post translational protein modification; protein polyubiquitination;
5	Serine/threonine-protein kinase 26	ATP binding; magnesium ion binding; protein homodimerization activity; protein kinase activity; protein serine/threonine kinase activity	activation of protein kinase activity; neuron projection morphogenesis; protein phosphorylation; signal transduction by protein phosphorylation
6	Angiopoietin-like protein 8	hormone activity	cell maturation; cellular lipid metabolic process; lipid metabolic process; lipoprotein lipase activity;
7	Plasma kallikrein	serine-type endopeptidase activity	Factor XII activation; blood coagulation, intrinsic pathway; extracellular matrix disassembly; fibrinolysis; plasminogen activation
8	ATP-citrate synthase	ATP binding; ATP citrate synthase activity; cofactor binding; metal ion binding	acetyl-CoA and cholesterol biosynthetic process; citrate metabolic process; coenzyme A metabolic process
9	ATP-binding cassette sub-family B member 6, mitochondrial	ATP binding; ATPase activity; ATPase-coupled heme transmembrane transporter activity; heme binding	cellular iron ion homeostasis; heme transport; transmembrane transport
10	ADP-ribosylation factor 6	GTP binding; GTPase activity; protein N-terminus binding; thioesterase binding	intracellular protein transport; maintenance of postsynaptic density structure; protein localization to endosome; synaptic vesicle endocytosis

NO.	Target protein	Molecular function	Biological process
11	60 kDa heat shock protein, mitochondrial	ATP binding; ATPase activity;apolipoprotein binding; chaperone binding	chaperone-mediated protein complex assembly; B cell activation and cytokine production; protein import into mitochondrial intermembrane space
12	Phospholipid scramblase 1	CD4 receptor binding; DNA binding transcription activator activity, calcium ion binding; phospholipid scramblase activity	apoptotic process; phosphatidylserine biosynthetic process; plasma membrane phospholipid scrambling
13	Calcium and integrin-binding protein 1	Ras GTPase binding; calcium ion binding; calcium dependent protein kinase inhibitor activity; protein C-terminus binding; protein kinase binding	Angiogenesis; apoptotic process; cell adhesion; cellular response to DNA damage stimulus and growth factor stimulus; cytoplasmic microtubule organization
14	Filaggrin-2	calcium ion binding; structural constituent of epidermis; transition metal ion binding	cell adhesion; epidermis morphogenesis; neutrophil degranulation
15	Mucin-16	thought to provide a protective, lubricating barrier against particles and infectious agents at mucosal surfaces	O-glycan processing; cell adhesion; stimulatory C-type lectin receptor signaling pathway
16	Integrin alpha-1	collagen binding; collagen binding involved in cell-matrix adhesion; metal ion binding; protein phosphatase binding; signaling receptor binding	activation of MAPK activity; neuron projection morphogenesis; neutrophil chemotaxis; neuron apoptotic process; phosphoprotein phosphatase activity; vasodilation
17	Zinc-alpha-2-glycoprotein	protein transmembrane transporter activity; ribonuclease activity	cell adhesion; detection of chemical stimulus involved in sensory perception of bitter taste; retina homeostasis; transmembrane transport
18	Prelamin-A/C	identical protein binding; structural molecule activity	protein localization to nucleus and protein stability; cellular response to hypoxia; nuclear envelope organization; telomere maintenance
19	class I histocompatibility antigen, B-48 alpha chain	peptide antigen binding	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent; antigen processing and presentation of exogenous peptide antigen
20	Solute carrier family 40 member 1	iron ion transmembrane transporter activity; peptide hormone binding	cellular iron ion homeostasis; iron ion export across plasma membrane; iron ion transmembrane transport; lymphocyte homeostasis; spleen trabecula formation

NO.	Target protein	Molecular function	Biological process
21	Interferon-induced transmembrane protein 1	inhibits the entry of viruses to the host cell cytoplasm, permitting endocytosis, but preventing subsequent viral fusion	cell surface receptor signaling pathway; response to interferon alpha or beta; type I interferon signaling pathway
22	Proteasome subunit alpha type-7	endopeptidase activity; identical protein binding; threonine-type endopeptidase activity	Ubiquitin dependent protein catabolic process; post translational protein modification; protein deubiquitination; protein polyubiquitination
23	Multifunctional protein ADE2	cadherin binding; phosphoribosylaminoimidazole carboxylase synthase activity	purine nucleobase biosynthetic process; purine ribonucleoside monophosphate biosynthetic process
24	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	protein antigen binding; protein heterodimerization activity; protein phosphatase regulator activity; protein serine/threonine phosphatase activity	RNA splicing; ceramide metabolic process; peptidyl serine dephosphorylation; protein dephosphorylation
25	Plexin-A4	semaphorin receptor activity	chemorepulsion of branchiomotor axon; regulation of axonogenesis, axon extension and GTPase activity; semaphorin-plexin signaling pathway involved in axon guidance
26	E3 ubiquitin-protein ligase TRIM58	dynein heavy chain binding ;dynein intermediate chain binding ;ubiquitin protein ligase activity; zinc ion binding	protein autoubiquitination; protein polyubiquitination; regulation of nuclear migration along microtubule; ubiquitin dependent protein catabolic process
27	Endoplasmic reticulum resident protein 44	protein disulfide isomerase activity	cell redox homeostasis; glycoprotein metabolic process; response to endoplasmic reticulum stress and unfolded protein

Table 7
The symbols and full names of the predicted functional intermediate partners in the PPI network of up-regulated expressed proteins shown in Fig. 7.

NO	Accession NO	Symbol	Full name	Molecular function	Number of amino acid
1	P25787	PSMA2	Proteasome subunit alpha type-2	component of the 20S core proteasome complex involved in the proteolytic degradation of most intracellular proteins.	234
2	P25789	PSMA4	proteasome subunit alpha type-4	participates in the ATP-dependent degradation of ubiquitinated proteins and plays a key role in the maintenance of protein homeostasis by removing misfolded or damaged proteins.	261
3	P16144	ITGB4	Integrin beta-4	binds to NRG1 (via EGF domain) and this binding is essential for NRG1-ERBB signaling	1822
4	P43686	PRS6B	26S proteasome regulatory subunit 6B	a multiprotein complex involved in the ATP-dependent degradation of ubiquitinated proteins.	418
5	P51665	PSMD7	26S proteasome non-ATPase regulatory subunit 7	plays a key role in the maintenance of protein homeostasis by removing misfolded or damaged proteins.	324
6	O75832	PSMD10	26S proteasome non-ATPase regulatory subunit 10	acts as a chaperone during the assembly of the 26S proteasome, specifically of the PA700/19S regulatory complex	226
7	Q9NZZ3	CHMP5	Charged multivesicular body protein 5	peripherally associated component of the endosomal sorting required for transport complex III which is involved in multivesicular bodies formation and sorting.	219
8	Q14242	SELPLG	P-selectin glycoprotein ligand 1	a SLe(x)-type proteoglycan, which through high affinity, calcium-dependent interactions, mediates rapid rolling of leukocytes over vascular surfaces in inflammation.	428

Table 8
The symbols and full names of the predicted functional intermediate partners in the PPI network of down-regulated expressed proteins shown shown in Fig. 8.

NO	Accession NO	Symbol	Full name	Molecular function	Number of amino acid
1	P22087	FBL	rRNA 2'-O-methyltransferase fibrillarin	S-adenosyl-L-methionine-dependent methyltransferase that has the ability to methylate both RNAs and protein, catalyzing the site-specific 2'-hydroxyl methylation of ribose moieties in pre-ribosomal RNA.	321
2	P14866	HNRNPL	Heterogeneous nuclear ribonucleoprotein L	splicing factor binding to exonic or intronic sites and acting as either an activator or repressor of exon inclusion, Exhibiting a binding preference for CA-rich elements.	589
3	P36954	POLR2I	DNA-directed RNA polymerase II subunit RPB9	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.	125
4	P62714	PPP2CB	Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	PP2A can modulate the activity of phosphorylase B kinase casein kinase 2, mitogen-stimulated S6 kinase, and MAP-2 kinase.	309
5	P25788	PSMA3	Proteasome subunit alpha type-3	plays numerous essential roles within the cell by associating with different regulatory particles.	255
6	P48556	PSMD8	26S proteasome non-ATPase regulatory subunit 8	component of the 26S proteasome and participates in apoptosis or DNA damage repair.	350
7	P62877	RBX1	E3 ubiquitin-protein ligase RBX1	E3 ubiquitin ligase component of multiple cullin-RING- based E3 ubiquitin-protein ligase (CRLs) complexes which mediate the ubiquitination and subsequent proteasomal degradation of target proteins.	108

3.4 GO analysis results of the differentially expressed proteins

A GO analysis was performed to classify the protein classification, molecular function, biological process, cellular component, and pathway.

3.4.1 GO analysis results of the up-regulated proteins

Protein classification results indicated that 7.0%, 5.5%, 4.7%, 4.7%, 5.5%, 3.9% and 4.7% of these 128 up-regulated proteins were cytoskeletal protein, membrane traffic protein, metabolite interconversion enzyme, protein modifying enzyme, protein-binding activity modulator, scaffold/adaptor protein, and transporter, respectively (Fig. 4A). Molecular function classification results indicated that 26.6%, 17.2%, 4.7% of these 128 up-regulated proteins were binding, catalytic activity, and transporter activity, respectively (Fig. 4B). In biological process classification, most of the proteins were determined to be involved in biological regulation (28.1%), cellular component organization or biogenesis (18.0%), cellular process (39.8%), localization (18.8%), metabolic process (15.6%), response to stimulus (21.9%), and signaling (16.4%), respectively (Fig. 4C). According to the classification of pathways, most of these proteins were related to blood coagulation (4.8%), CCKR signaling map (3.1%), EGF receptor signaling pathway (3.1%), endothelin signaling pathway (3.9%), FGF signaling pathway (3.1%), inflammation mediated by chemokine and cytokine signaling pathway (7.0%), integrin signalling pathway (6.3%), PI3 kinase pathway (2.3%) (Fig. 5). The blood coagulation pathway included proteinase-activated receptor 4, platelet glycoprotein V, integrin alpha- b, platelet glycoprotein Ib alpha chain, platelet glycoprotein Ib beta chain, and platelet glycoprotein IX. The endothelin signaling pathway included endothelin-converting enzyme 1, cAMP-dependent protein kinase catalytic subunit beta, protein kinase C beta type, mitogen-activated protein kinase 1, and guanine nucleotide-binding protein subunit alpha-14.

3.4.2 GO analysis results of the down-regulated proteins

Protein classification results indicated that 5.9%, 5.9%, 8.5%, 3.4%, 10.2% and 4.2% of these 118 down-regulated proteins were cytoskeletal protein, extracellular matrix protein, metabolite interconversion enzyme, nucleic acid binding protein, protein modifying enzyme, and protein-binding activity modulator, respectively (Fig. 6A). Molecular function classification results indicated that 28.2%, 24.8%, 5.1%, 2.6% and 5.1% of these down-regulated proteins were binding, catalytic activity, molecular function regulator, molecular transducer activity and transporter activity, respectively (Fig. 6B). In biological process classification, most of the proteins were determined to be involved in biological regulation (20.3%), cellular component organization or biogenesis (18.6%), cellular process (39.0%), immune system process (6.8%), localization (13.6%), metabolic process (21.2%), multicellular organismal process (9.3%), response to stimulus (16.1%) and signaling (9.3%) (Fig. 6C). According to the classification of pathways, most of these proteins were related to gonadotropin-releasing hormone receptor pathway (1.7%), inflammation mediated by chemokine and cytokine signaling pathway (3.4%), integrin signalling pathway (3.4%) and ubiquitin proteasome pathway (1.7%) (Fig. 7). The inflammation mediated by chemokine and cytokine signaling pathway included rho-related GTP-binding protein, platelet factor 4 variant, C5a anaphylatoxin chemotactic receptor 1, and C-X-C chemokine receptor type 2. The integrin signalling pathway included collagen alpha-1(I) chain, collagen alpha-2(I) chain, integrin alpha-1, and ADP-ribosylation factor 6.

3.5 PPI networks analysis results

3.5.1 PPI networks analysis results of the up-regulated proteins

STRING analysis was performed to construct a high-quality controlled PPI network. 127 up-regulated proteins proteins were eligible for PPI network analysis (focus molecule) and a high-quality PPI networks were built based on the STRING database. A comprehensive PPI regulation network by 127 up-regulated proteins were shown in Fig. 8. The network clustering results revealed that the PPI network was consist of six specified function clusters which contain similar function proteins and are represented by different colors (Fig. 9). These six visualized interaction function clusters (sub-networks) were related to degradation of ubiquitinated proteins and folding of proteins (lime green), calcium-dependent protein binding and ESCRT III complex disassembly (yellow), cytoskeleton reorganization and platelet aggregation (green), phospholipid scrambling of phosphatidylserine in platelets and ATP mediates synaptic transmission (purple), lipid binding, signal transduction (red), and blood coagulation, intrinsic pathway (blue), respectively. The predicted functional intermediate partners in the PPI network are shown in table.7.

3.5.2 PPI networks analysis results of the down-regulated proteins

118 down-regulated proteins were eligible for PPI network analysis (focus molecule) and a high-quality controlled PPI networks were built based on the STRING database. A comprehensive PPI regulation network by down-regulated proteins were shown in Fig. 10. The network clustering result revealed that the PPI network was consist of six specified function clusters which contain similar function proteins and are represented by different colors (Fig. 11). These six visualized interaction function clusters (sub-networks) were related to protein localization to juxtaparanode region of axon (yellow), cell adhesive protein binding, formation of Fibrin Clot (green), mRNA splicing and RNA recognition (red), complement activation, lipid metabolism (purple), protein trafficking and cytoskeleton remodeling (lime green), and ATP-dependent degradation of ubiquitinated proteins (blue), respectively. The predicted functional intermediate partners in the PPI network are shown in table.8.

3.6 Quantitative determination of plasma proteins

Compared with control group, plasma levels of human 14-3-3 protein zeta/delta (YWHAZ) and human brain-specific angiogenesis inhibitor 1 associated protein 2 (BAIAP2) BAIAP2 were significantly increased ($P < 0.01$) while that of human IgD (IGHD), human ATP binding cassette subfamily B member 6, mitochondrial (ABCB6) and human heat shock protein 60 (HSPD1) were significantly decreased in patients with and without post stroke cognitive impairment ($P < 0.01$) (Fig. 12).

4. Discussion

4.1 General Comments

To further explore the molecular mechanism of cognitive impairment, label-free quantitative proteomics were employed to analyse the differential expressed proteins of plasma exosome in PSCI patients. Proteomics identificated 259 differentially expressed proteins, including 131 up-regulated proteins and 128 down-regulated proteins. These up-regulated proteins are related to degradation of ubiquitinated proteins, calcium dependent protein binding, cytoskeleton reorganization and platelet aggregation and blood coagulation. These down-regulated proteins are related to protein localization to juxtaparanode region of axon, cell adhesive protein binding, formation of fibrin clot, complement activation, lipid metabolism and ATP-dependent degradation of ubiquitinated proteins. The mechanisms of cognitive impairment of PSCI are related to blood flow regulation, energy metabolism, protein folding and degradation, cell apoptosis, synaptic plasticity. These were discussed in detail below.

4.2 Blood flow regulation associated proteins

Plasma kallikrein, a multifunctional serine protease involved in contact activation of coagulation(12). The mechanisms of action of plasma kallikrein can be used to support pro-thrombotic or anti-thrombotic effects. The kallikreinkinin system inhibits thrombin-induced platelet activation, suggesting an anti-thrombotic role(13). Plasma kallikrein reduced collagen-induced platelet activation by binding collagen (14). In contrast, a pro-thrombotic effect is suggested by the important role of plasma kallikrein in contact activation via conversion of FXII to FXIIa. Plasma kallikrein also converts prorenin to renin which converts angiotensinogen to angiotensin I(15). Plasma kallikrein had been implicated in contributing to both hematoma expansion and thrombosis in stroke(16). The results of this study showed that the expression of plasma kallikrein was down-regulated. Plasma kallikrein may influence the occurrence and development of acute stroke through the activation and transformation pathway of FXII.

Platelet glycoprotein V (gpV), a membrane constituent with a relative molecule mass of 82 kDa, belongs to the leucine-rich family of proteins. Its expression is restricted to platelets and megakaryocytes, and is non-covalently linked to the gpIb-IX complex to form a receptor for von Willebrand factor and thrombin (17, 18). The GPIb-V-IX complex functions as the vWF receptor and mediates vWF-dependent platelet adhesion to blood vessels. The adhesion of platelets to injured vascular surfaces in the arterial circulation is a critical initiating event in hemostasis(19). Platelet glycoprotein V may serve as an in vivo marker of platelet activation in thrombotic states. The expression of platelet-glycoprotein V in patients with acute stroke is increased, which is consistent with the study of Amin HM et al(20). Therefore, platelet glycoprotein V may play a protective role in the brain through blood coagulation. Fibrinogen like protein 1 (FGL1) is a secreted protein with mitogenic activity on primary hepatocytes. FGL1 contains an N-terminal signal recognition peptide, a potential N-terminal coil-coil domain, a C-terminal fibrinogen related domain (FReD) and multiple cysteines presumably used for inter and intra molecular disulfide bonds(21). FGL1 may play a potential role in these processes including angiogenesis, proliferation, apoptosis and extracellular matrix modulation like structurally related proteins (angiopoietins, tenascins, fibrinogen)(22, 23). Additionally, the presence of FGL1 in rat serum following cytokine stimulation suggests that it may serve as a biological marker for systemic inflammation(24). Therefore, FGL1 may mediate the inflammatory response directly or indirectly in acute stroke. Our result of the increased expression of this protein just confirms this hypothesis.

4.3 Energy Metabolism

ATP-binding cassette sub-family B member 6 (ABCB6), a member of the adenosine triphosphate–binding cassette (ABC) transporter family. It binds heme and porphyrins and functions in their ATP-dependent uptake into the mitochondria and plays a crucial role in heme synthesis(25). Some studies demonstrated that ABCB6 expression was protective against different insults increasing oxidative stress, such as arsenite exposure (26, 27). The results of this study showed that the expression level of ABCB6 decreased in patients with acute stroke. The expression and function of ABCB6 are closely related to the oxidation mechanism of mitochondria(25). Therefore, ABCB6 is likely to play a protective role in the brain through antioxidant mechanisms.

4.4 Synaptic plasticity

Brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2), adapter protein that links membrane-bound small G-proteins to cytoplasmic effector proteins. Subsequent studies have firmly established BAIAP2 as an important regulator of membrane and actin dynamics at actin-rich subcellular structures, including filopodia and lamellipodia(28–30). Actin skeleton and its dynamics play an important role in the regulation of excitatory synaptic transmission and plasticity (31, 32). The results of this study showed that the expression level of BAIAP2 increased in patients with acute stroke. In the brain, BAIAP2 may play a neuroprotective role by improving synaptic function.

5. Conclusion

In conclusion, the present study found 259 differentially expressed proteins including 131 up-regulated proteins and 128 down-regulated proteins using label-free quantitative proteomics approach in plasma exosome of PSCI patients. The findings suggest that the mechanism of cognitive impairment may be related to blood flow regulation, energy metabolism, protein folding and degradation, cell apoptosis, synaptic plasticity, stress response and protein phosphorylation in PSCI patients. Therefore, these proteins might be target-related proteins and provide global insights into pathogenesis mechanisms of cognitive impairment at plasma exosome proteins level in PSCI patients.

Declarations

Acknowledgments

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository(33) with the dataset identifier PXD028200.

Authors' contributions

Qi BY and Wei DF designed the research; Qi BY, Wei DF, Kong LB, Lai XX, Wang LS, Fei Liu F and Ji WW performed research and analyzed data; Qi BY and Wei DF wrote the manuscript; Qi BY, Wei DF and Ji WW revised the manuscript. The final manuscript was reviewed and approved by all authors.

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Ethics Approval and Consent to Participate

Not applicable.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Dongdong W, Yanfeng G, Yuanming Q, Lixiang C, Yuanfang M, Yanzhang L. Peptide-based cancer therapy: opportunity and challenge. Cancer Letters 2014;351(1):13–22.
2. Shyam P, Ilana R, Bernstein RA. Acute stroke intervention: a systematic review. Journal of the American Medical Association 2018;313(14):1–14.
3. György B, Hung ME, Breakefield XO, Leonard JN. Therapeutic Applications of Extracellular Vesicles: Clinical Promise and Open Questions. Annu Rev Pharmacol Toxicol 2015;55(1):439–64.
4. Li DB, Liu JL, Wang W, et al. Plasma Exosomal miRNA-122-5p and miR-300-3p as Potential Markers for Transient Ischaemic Attack in Rats. Frontiers in Aging Neuroscience 2018;10:24-.
5. Haqqani AS. Method for isolation and molecular characterization of extracellular microvesicles released from brain endothelial cells. Fluids & Barriers of the Cns 2013;10(1):4-.
6. Yanlu Z, Michael C, Yuling M, et al. Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. Journal of Neurosurgery 2015;122(4):856–67.
7. Hongqi X, Yi L, Yisheng C, Yang JJ, Gang ZZ, Michael C. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. Journal of Cerebral Blood Flow & Metabolism Official Journal of the International Society of Cerebral Blood Flow & Metabolism 2013;33(11):1711–5.
8. Arba F, Quinn T, Hankey GJ, Ali M, Lees KR, Inzitari D. Cerebral small vessel disease, medial temporal lobe atrophy and cognitive status in patients with ischaemic stroke and transient ischaemic attack. Eur J Neurol 2017;24(2):276–82.

9. Tu J, Wang LX, Wen HF, Xu YC, Wang PF. The association of different types of cerebral infarction with post-stroke depression and cognitive impairment. Medicine 2018;97(23):e10919.
10. Gao F, Jiao F, Xia C, et al. A novel strategy for facile serum exosome isolation based on specific interactions between phospholipid bilayers and TiO2. Chem Sci 2019;10(6):1579–88.
11. Szklarczyk D, Morris JH, Cook H, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic acids research 2017;45(D1):D362-D8.
12. Bird JE, Smith PL, Wang X, Schumacher WA, Seiffert D. Effects of plasma kallikrein deficiency on haemostasis and thrombosis in mice: Murine Ortholog of the Fletcher Trait. Thromb Haemost 2012;107(6):1141–50.
13. Schmaier AH, Smith PM, Purdon AD, White JG, Colman RW. High molecular weight kininogen: localization in the unstimulated and activated platelet and activation by a platelet calpain(s). Blood 2013;67(1):119–30.
14. Liu J, Gao B-B, Clermont AC, et al. Hyperglycemia-induced cerebral hematoma expansion is mediated by plasma kallikrein. Nature Medicine 2011;17(2):206–10.
15. Ceravolo GS, C. MA, T. JoM, et al. An Interaction of Renin-Angiotensin and Kallikrein-Kinin Systems Contributes to Vascular Hypertrophy in Angiotensin II-Induced Hypertension: In Vivo and In Vitro Studies. Plos One 2014;9(11):e111117-.
16. Simão F F, Ustunkaya T, Clermont AC, Feener EP. Plasma kallikrein mediates brain hemorrhage and edema caused by tissue plasminogen activator therapy in mice after stroke. Blood 2017;129(16):2280–90.
17. Lanza F, Morales M, De LSC, et al. Cloning and characterization of the gene encoding the human platelet glycoprotein V. A member of the leucine-rich glycoprotein family cleaved during thrombin-induced platelet activation. Journal of Biological Chemistry 1993;268(28):20801.
18. Hickey MJ, Hagen FS, Yagi M, Roth GJ. Human Platelet Glycoprotein V: Characterization of the Polypeptide and the Related Ib-V-IX Receptor System of Adhesive, Leucine-Rich Glycoproteins. Proceedings of the National Academy of Sciences of the United States of America 1993;90(18):8327–31.
19. Moog S. Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation. Blood 2001;98(4):1038–46.
20. Amin HM, Ahmad S, Walenga JM, Hoppensteadt DA, Leitz H, Fareed J. Soluble P-Selectin in Human Plasma: Effect of Anticoagulant Matrix and its Levels in Patients With Cardiovascular Disorders. Clinical & Applied Thrombosis/hemostasis Official Journal of the International Academy of Clinical & Applied Thrombosis/hemostasis 2000;6(2):71–6.
21. Demchev V, Malana G, Vangala D, et al. Targeted deletion of fibrinogen like protein 1 reveals a novel role in energy substrate utilization. PloS one 2013;8(3):e58084.
22. Procopio WN, Pelavin PI, Lee WM, Yeilding NM. Angiopoietin-1 and – 2 coiled coil domains mediate distinct homo-oligomerization patterns, but fibrinogen-like domains mediate ligand activity. The Journal of biological chemistry 1999;274(42):30196–201.

23. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood 2000;96(12):3772–8.
24. El-Karef A, Yoshida T, Gabazza EC, et al. Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. J Pathol 2007;211(1):86–94.
25. Kiss K, Brozik A, Kucsma N, et al. Shifting the paradigm: the putative mitochondrial protein ABCB6 resides in the lysosomes of cells and in the plasma membrane of erythrocytes. PLoS One 2012;7(5):e37378.
26. Chavan H, Oruganti M, Krishnamurthy P. The ATP-Binding Cassette Transporter ABCB6 Is Induced by Arsenic and Protects against Arsenic Cytotoxicity. Toxicological Sciences An Official Journal of the Society of Toxicology 2011;120(2):519–28.
27. Lynch J, Fukuda Y, Krishnamurthy P, Du G, Schuetz JD. Cell Survival under Stress Is Enhanced by a Mitochondrial ATP-Binding Cassette Transporter That Regulates Hemoproteins. Cancer Research 2009;69(13):5560–7.
28. Ahmed S, Goh WI, Bu W. I-BAR domains, IRSp53 and filopodium formation. Seminars in Cell and Developmental Biology 2010;21(4):0–356.
29. Scita G, Confalonieri S, Lappalainen P, Suetsugu S. IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions. Trends in Cell Biology 2008;18(2):52–60.
30. Suetsugu S, Toyooka K, Senju Y. Subcellular membrane curvature mediated by the BAR domain superfamily proteins. Seminars in Cell & Developmental Biology 2010;21(4):0–349.
31. Calabrese, B. Development and Regulation of Dendritic Spine Synapses. Physiology 2006;21(1):38–47.
32. Cingolani LA, Goda Y. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nature Reviews Neuroscience 2008;9(5):344–56.
33. Ma J, Chen T, Wu S, et al. iProX: an integrated proteome resource. Nucleic Acids Res 2019;47(D1):D1211-D7.

Figures

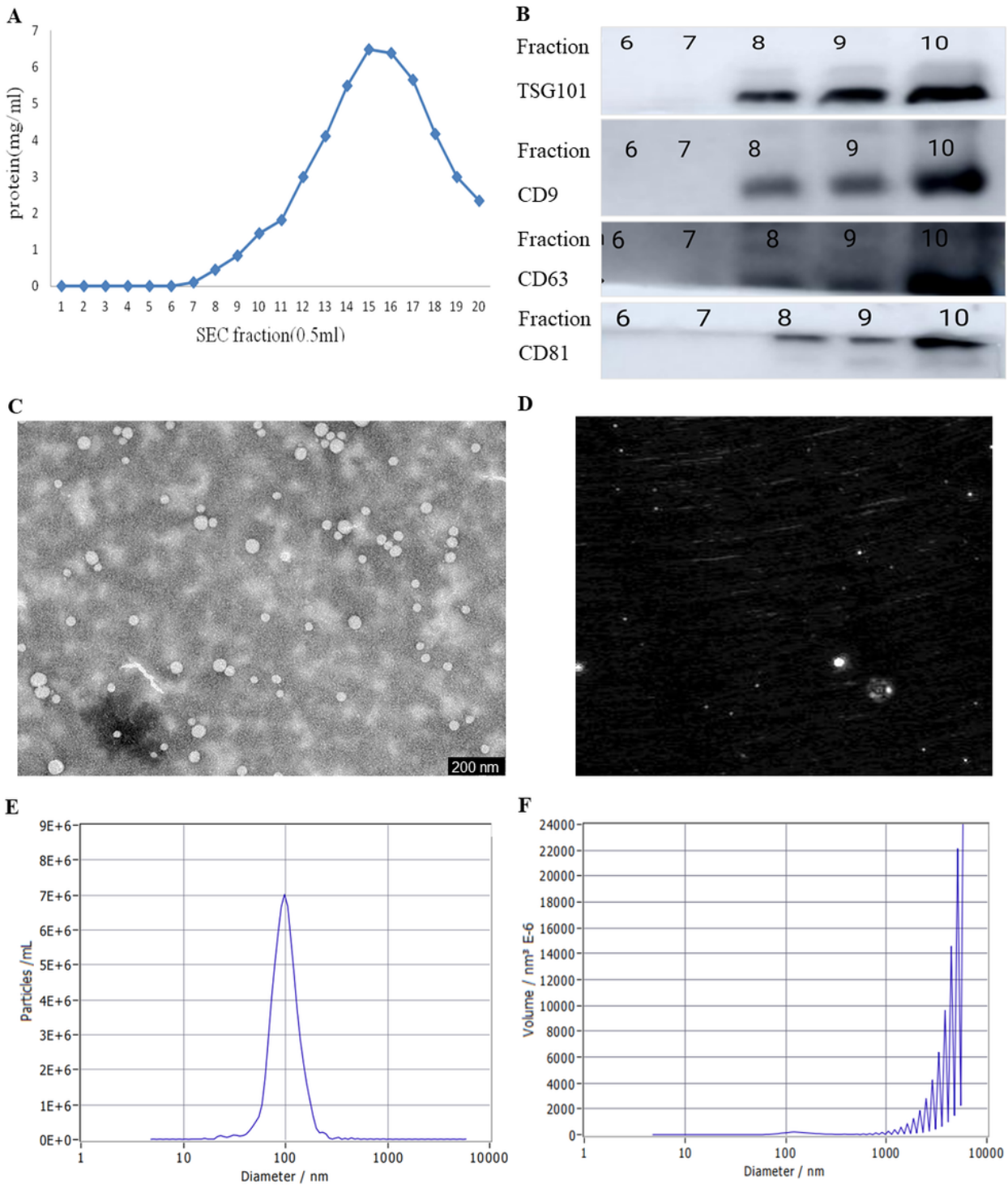

Figure 1

Various characterizations of plasma exosomes. (A) The content of plasma proteins in 0.5 mL fraction sample. (B) Western blot analysis of the typical exosomal proteins, TSG 101, CD9, CD63 and CD81. (C) Transmission electron microscopy (TEM) images indicating exosome morphology. (D) Dynamic light scattering (DLS) analysis of exosomes. (E) The Nanoparticle tracking analysis (NTA) result of the particle size distribution for isolated exosomes exosomes. (F) The size distribution of volume consistent with the size range of exosomes.

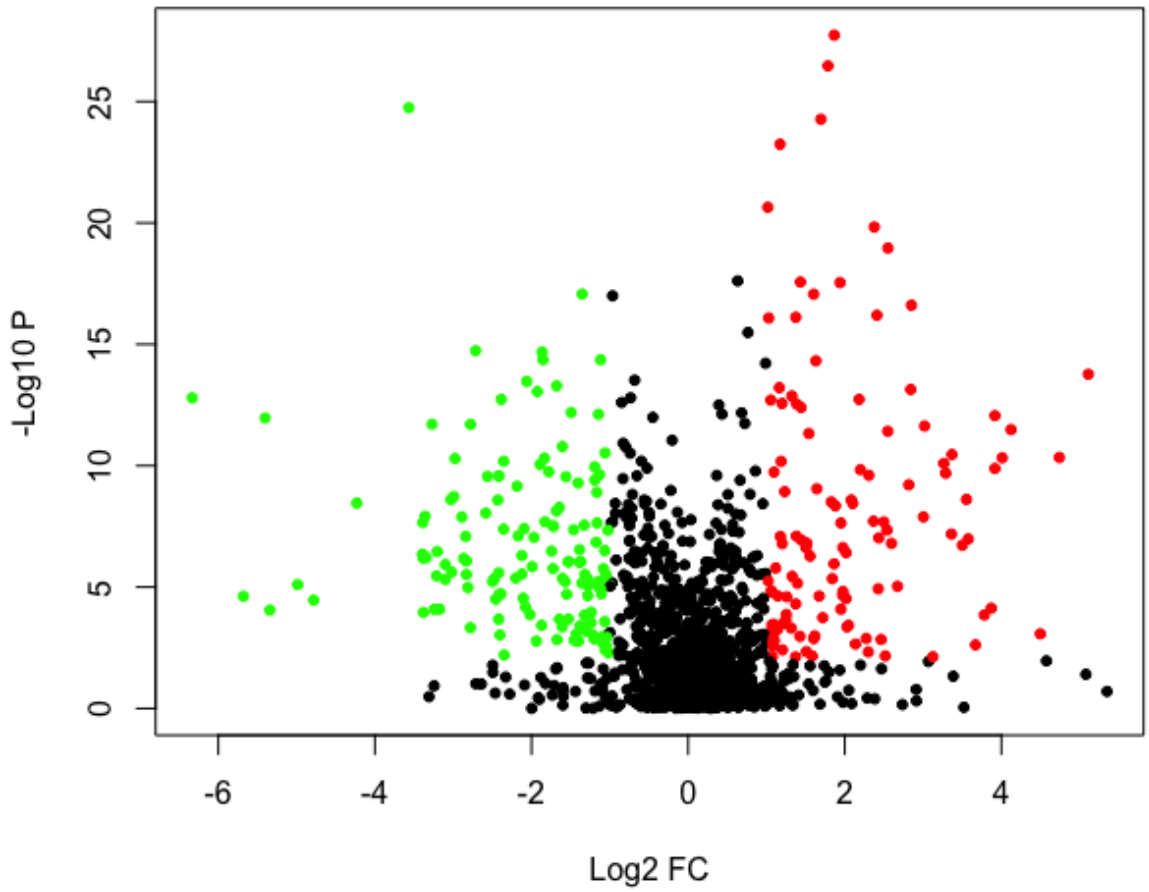

Figure 2

The Volcano plot of differentially expressed proteins. The red points represented up-regulated proteins and gree points represented down-regulated proteins between the PSCI and control groups.

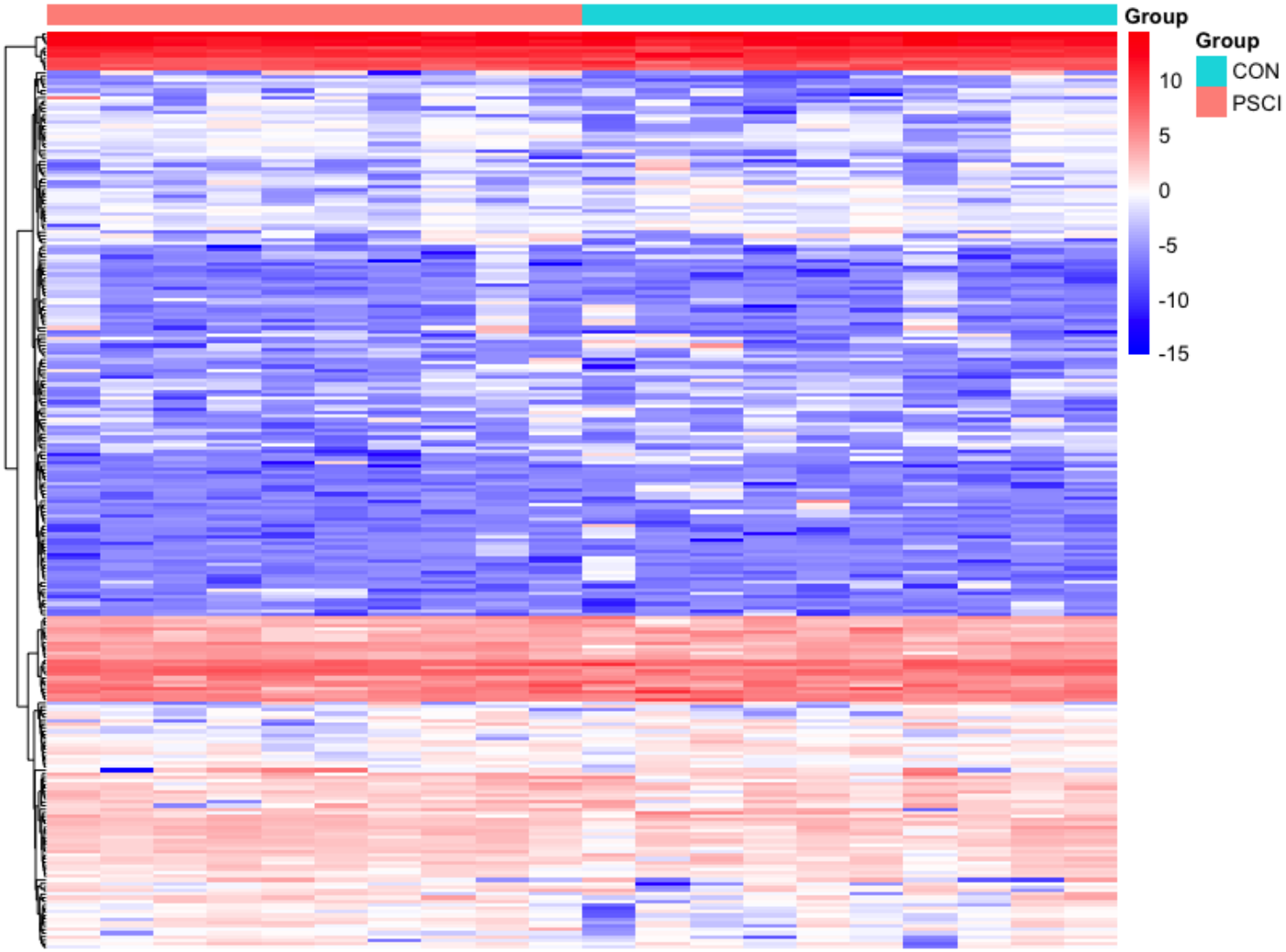

Figure 3

Hierarchical clustering of plasma exosome proteomes. The heat map represented the Z scores of all proteins quantified in Label-free quantitative proteomics.

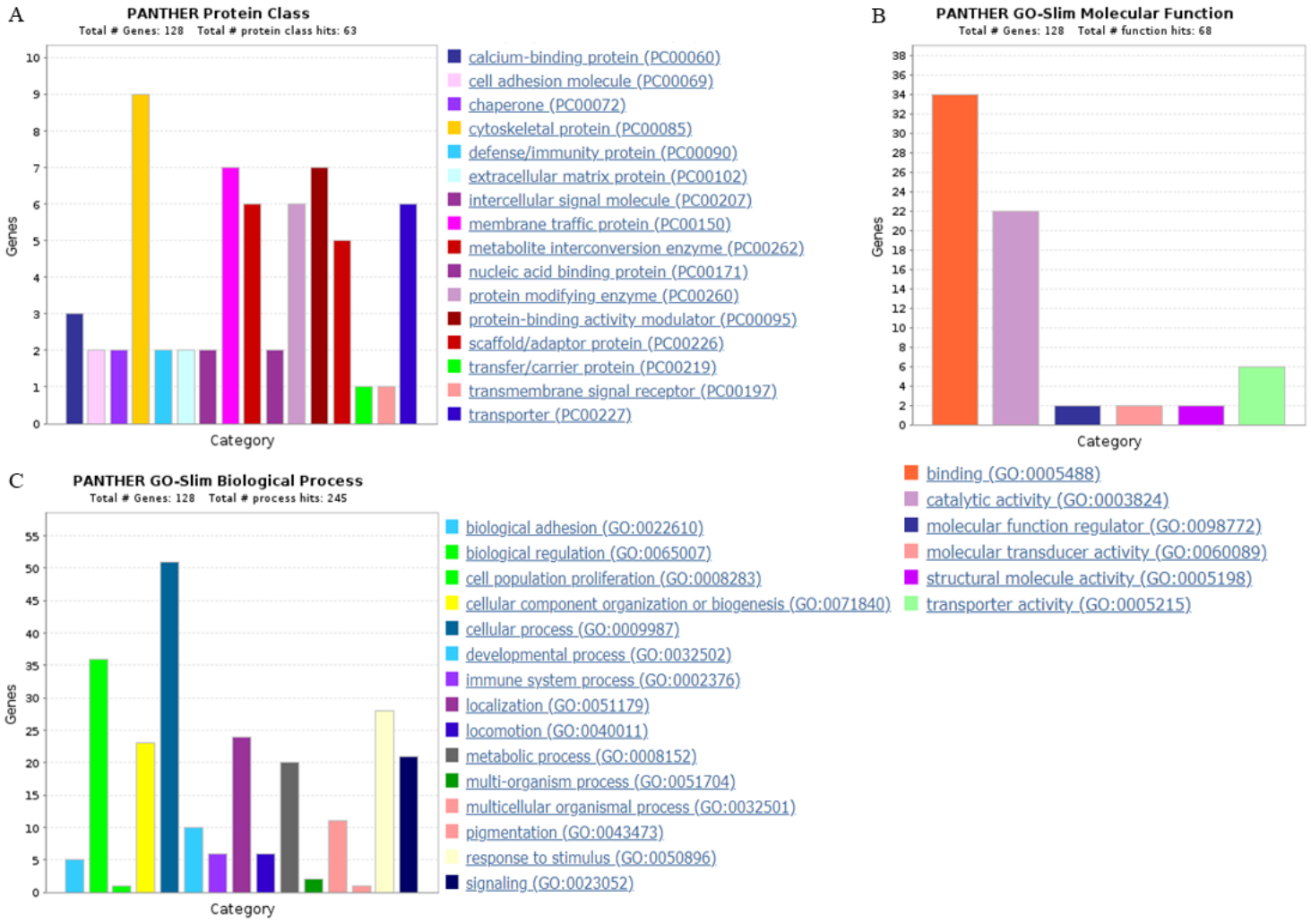

Figure 4

The protein classification, molecular function and biological process of up-regulated proteins in the PSCI group.

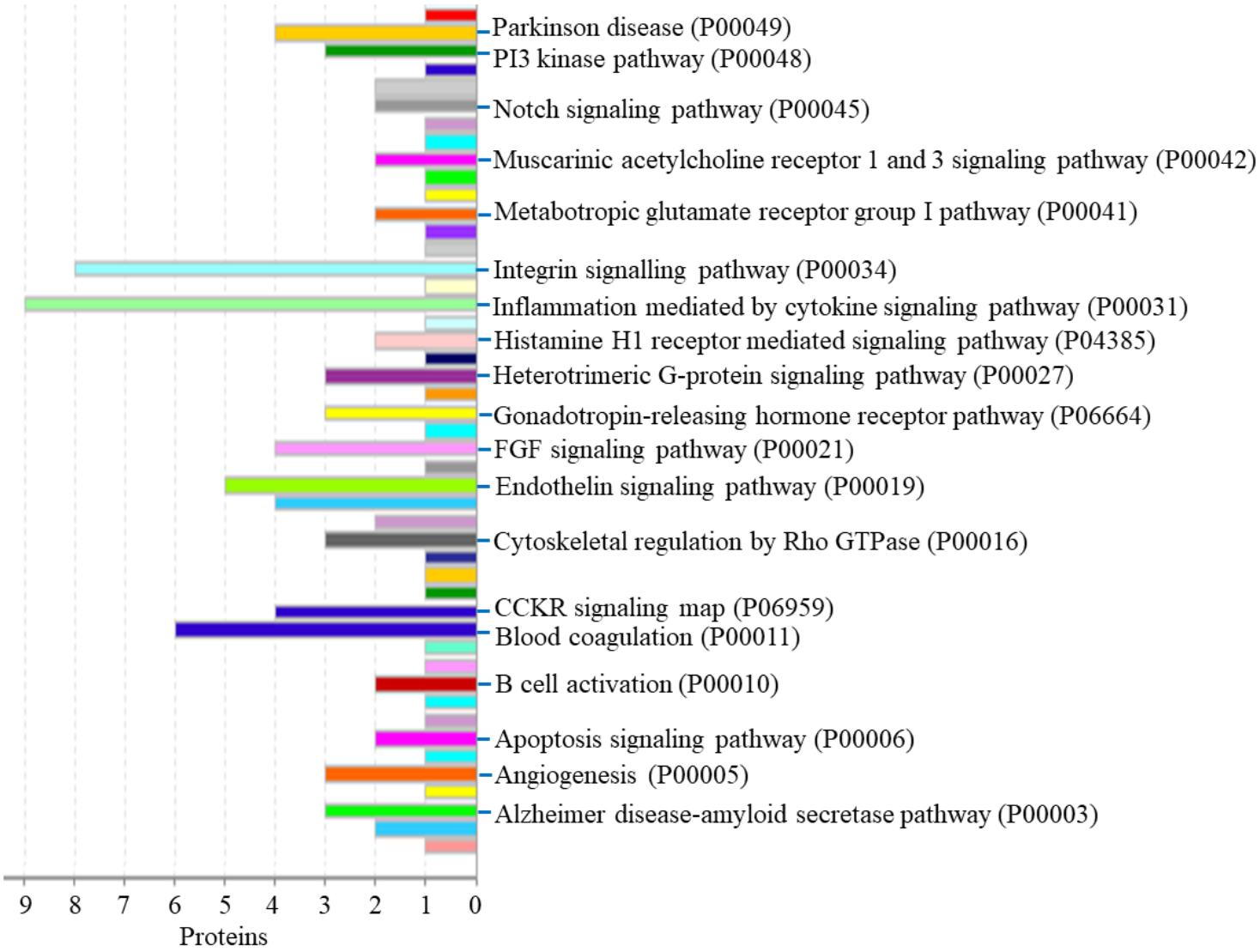

Figure 5

The pathway of up-regulated proteins in the PSCI group.

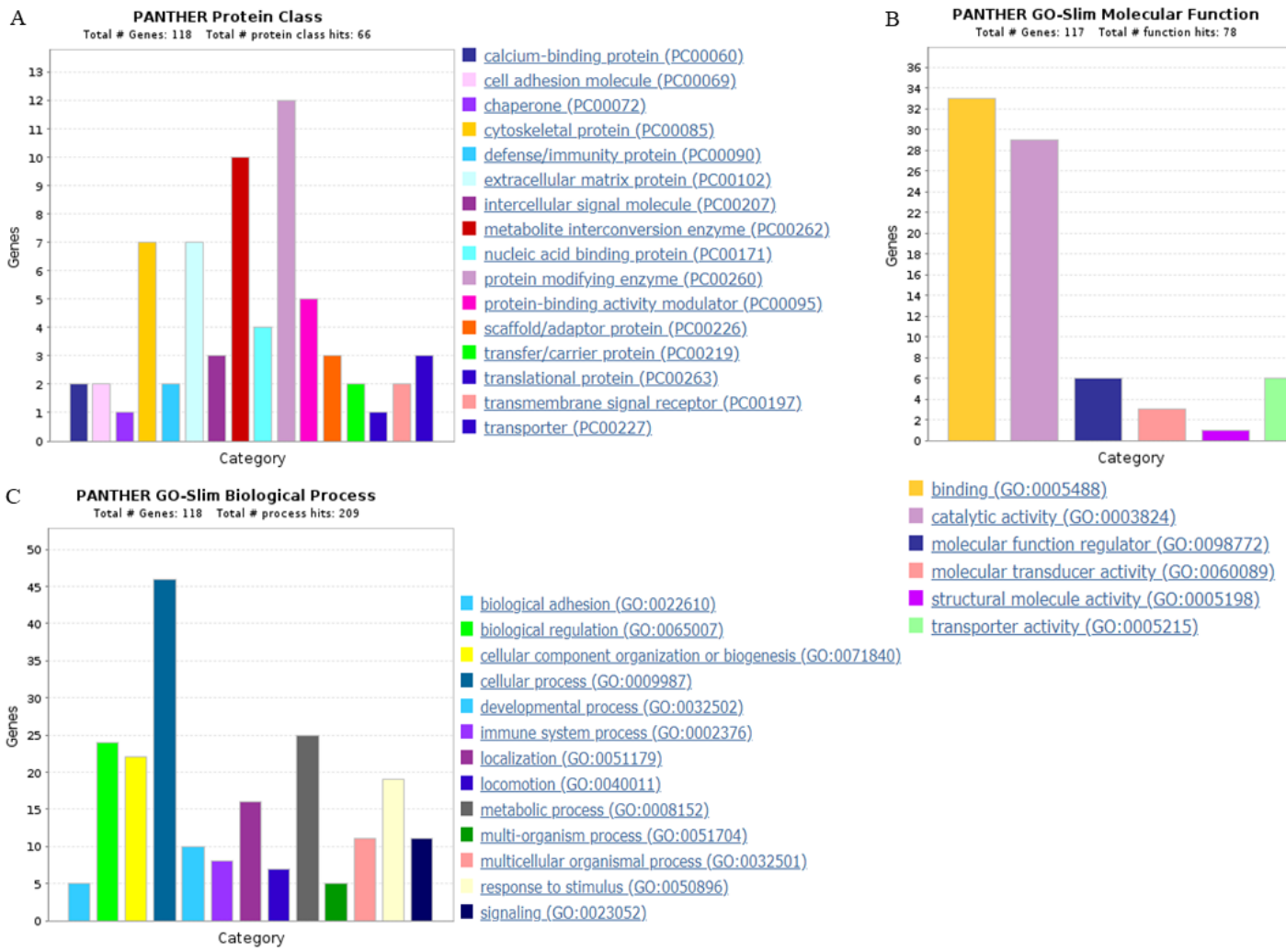

Figure 6

The protein classification, molecular function and biological process of down-regulated proteins in the PSCI group.

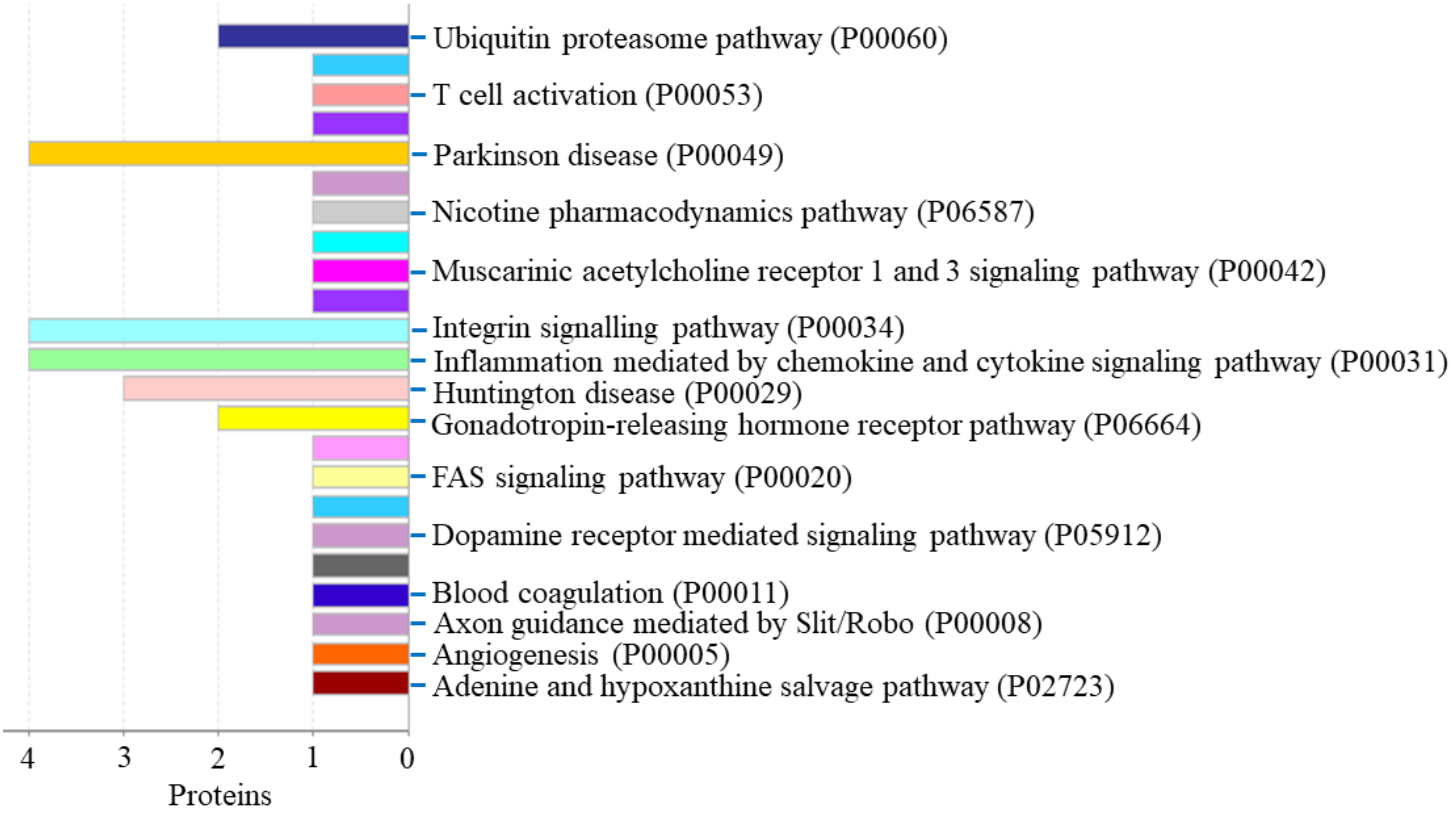

Figure 7

The pathway of down-regulated proteins in the PSCI group.

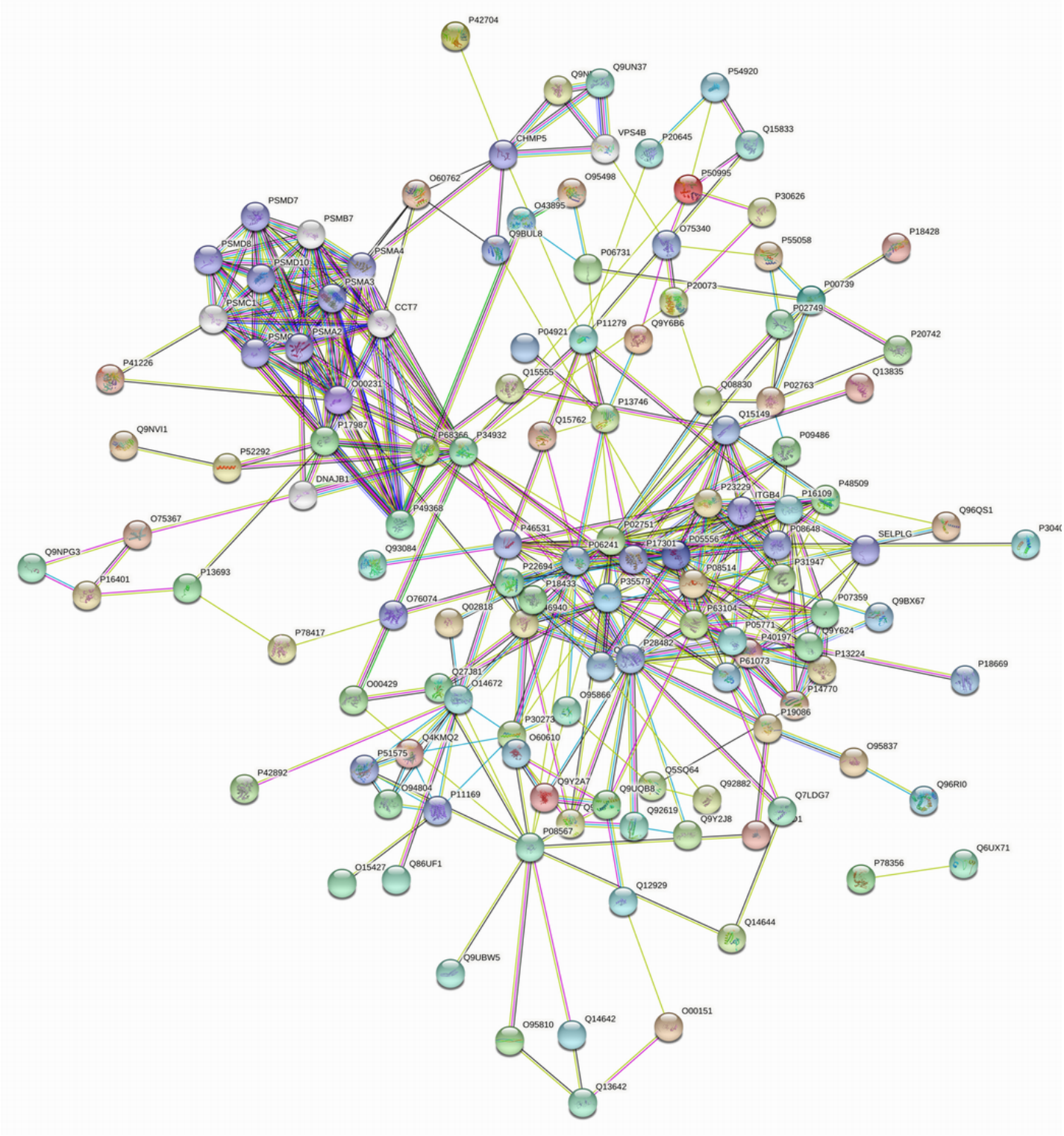

Figure 8

The PPI regulation network of up-regulated proteins in the PSCI group.

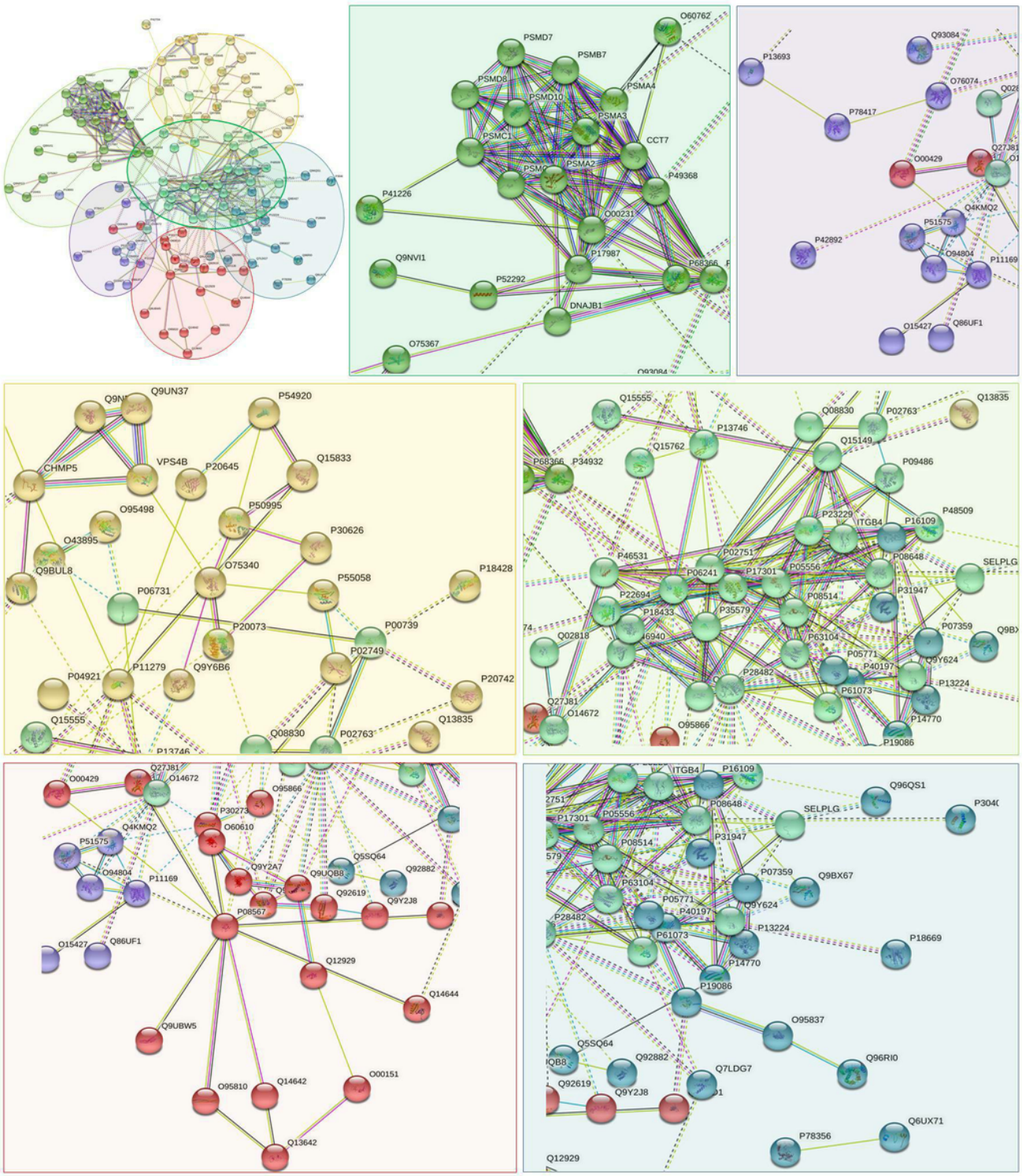

Figure 9

The PPI network kmeans clustering of up-regulated proteins in the PSCI group. The PPI network is clustered to a specified number of clusters.

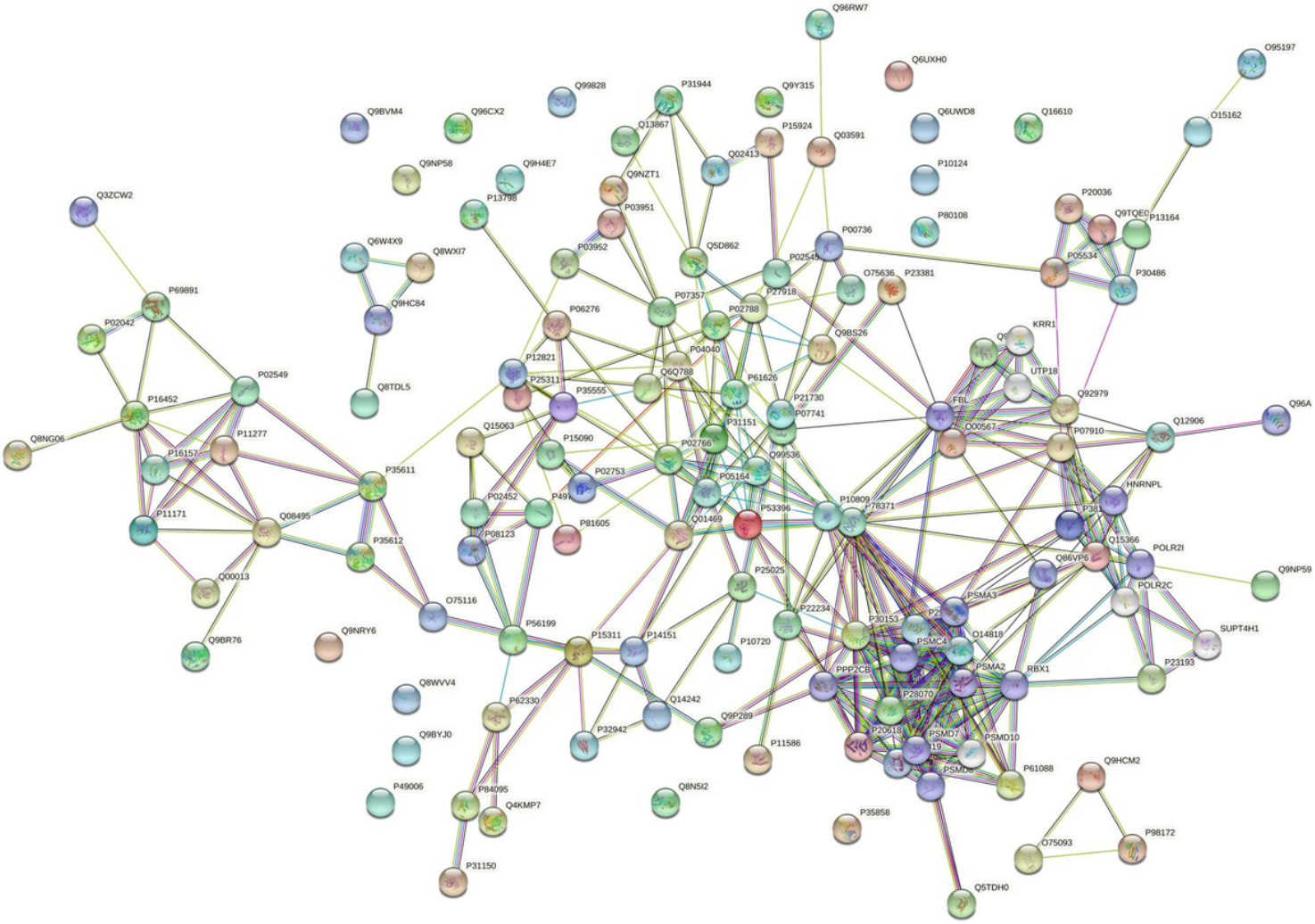

Figure 10

The PPI regulation network of down-regulated proteins in the PSCI group.

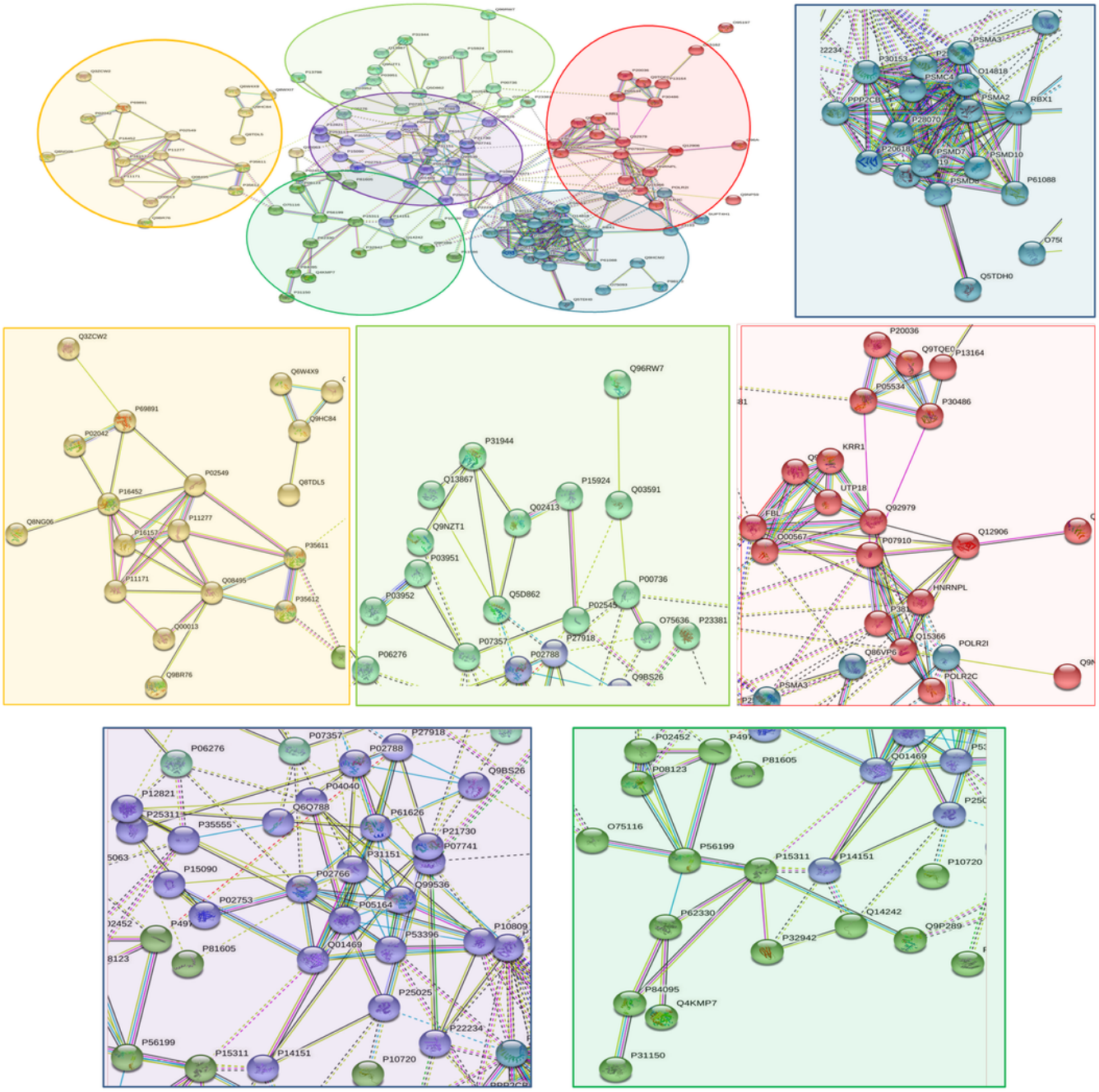

Figure 11

The PPI network kmeans clustering of down-regulated proteins in the PSCI group. The PPI network is clustered to a specified number of clusters.

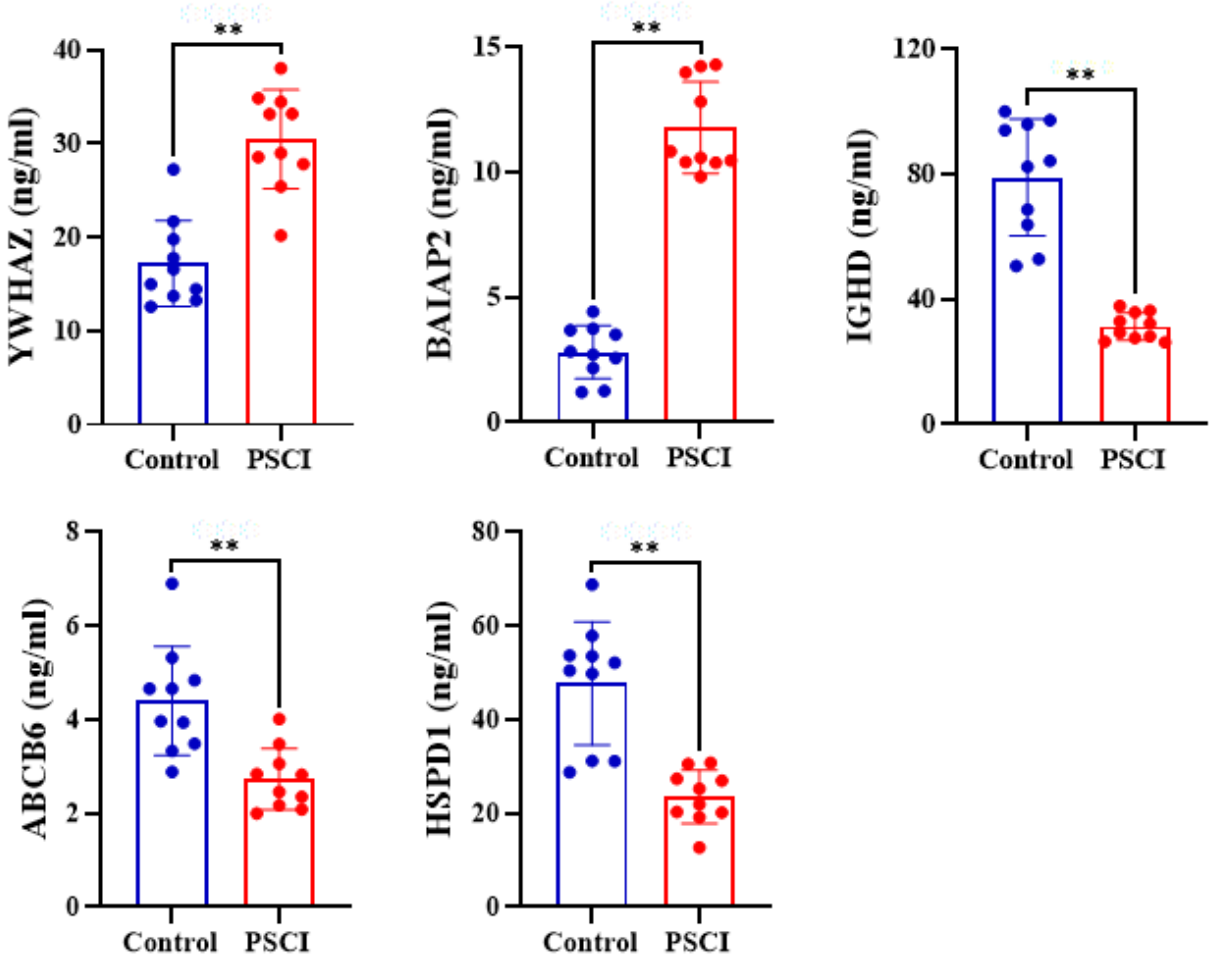

Figure 12

Plasma levels of human 14-3-3 protein zeta/delta (YWHAZ), human Brain-Specific Angiogenesis Inhibitor 1-Associated Protein 2 (BAIAP2), human IgD (IGHD), human ATP Binding Cassette Subfamily B Member 6, Mitochondrial (ABCB6), and human Heat Shock Protein 60 HSPD1 in patients with and without post stroke cognitive impairment.

Supplementary Files

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